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Real Time RT-PCR for tracing and quantification of Borna Disease Virus RNA in diseased hosts compared to experimentally inoculated ticks

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1. Abstract

Borna Disease is a severe, immunopathological disorder of the central nervous system (CNS), caused by infection with Borna Disease virus (BDV). The main known naturally affected animal species are horses and sheep in endemic areas in central Europe. The detection of BDV in these hosts is achieved by histological, immunohistochemical and serological approaches and/or PCR-based technologies.

In this study, we present the successful establishment of TaqMan® Real Time RT-PCR for the detection of the transcripts of the two major proteins, p40 (nucleoproteinprotein) and p24 (phosphoprotein). The primers detect BDV with high specificity and the approach also proved to be highly sensitive to detect both, plasmids containing the *p40* ORF and BDV from infected MDCK cells. In addition, Real Time RT-PCR allowed the relative quantification of virus load from different sheep and horse brain sites. These data were in complete agreement with previous immunohistochemical studies using the same samples and therefore demonstrated that the TaqMan® PCR approach is a valuable diagnostic and epidemiologic tool, which allows for comparative studies using infected samples from various sources, e.g., different tissues, body fluids and excretions (i.e., liquor, serum, urine). In addition, we tested the hypothesis whether the tick species *Ixodes ricinus* is a potential vector for the transmission of BDV. Our results show, that BDV infected cells could be detected over a period of one trough 24 days post feeding. However, in the course of time the virus load per tick decreased close to background. Our observations do not necessarily argue for or against ticks being vectors for BDV, but the experiments indicate that the newly established Real Time RT-PCR may provide a useful tool for detecting such vectors in endemic areas.

2. Introduction

Borna Disease (BD), a severe, immunopathological disorder of the central nervous system (CNS), is caused by infection with Borna Disease virus (BDV) and the main naturally affected animal species are horses and sheep in well circumscribed endemic areas in central Europe.

2.1. History

Attention attracted by a disease of horses now known to be associated with BDV, dates back several hundred years. The oldest reports describing typical characteristics of BD are found in 1660, speaking of pain which pushes the horses or makes them dull and dumb [1]. In 1716, a book was published recording sleepiness, melancholia and agitation in the course of such head pain [2]. More precise reports of a disease affecting the central nervous system in horses, called „disease of the head", was found later in the 18th and at the beginning of the 19th century [3,4]. The disease became more important in 1895 when almost all horses of a cavalry regiment in the town of Borna in Saxony near Leipzig showed the typical symptoms of a severe disease of the central nervous system [5]. After this time point the name Borna Disease was adopted. The famous investigations by Joest and Degen demonstrated that the disease was paralleled by inflammatory reactions. Furthermore, they discovered the pathognomonic appearance of intranuclear inclusion bodies, named Joest-Degen bodies [6]. The nature of the causing virus had remained obscure until the etiology of BD had been established as early as 1925, when Zwick and Seifried proved it's transmissibility [7]. More recently, BDV has been cultivated in different cell cultures [8,9], including MDCK cells, and aspects of pathogenesis of the disease have been elucidated [10,11]. Furthermore the molecular characterisation of BDV [12-15] and its replication strategy [16,17] have been achieved.

2.2. Etiology

2.2.1. Taxonomy and Morphology

BDV represents the only member of the new virus family *Bornaviridae* within the order *Mononegavirales*. This order also includes *Filoviridae* (e.g., Marburg and Ebola viruses), *Paramyxoviridae* (e.g., mumps, measles virus), and *Rhabdoviridae* (e.g., rabies, vesicular stomatitis virus) (see table 1). Electron microscopic studies of negative-stained cell-free BDV infectious particles have shown that they are of spherical morphology with a diameter ranging from 70 to 130nm [18-20]. These particles contain an internal electron-dense core (50 to 60nm) and a limiting outer membrane envelope, which appears to be covered with spikes approximately 7.0nm long [20]. Virus infectivity is rapidly lost by treatment at 56°C, as well as at pH's below 5 and above 12, and by treatment with organic solvents, detergents, formaldehyde and exposure to ultra violet radiation [21].

Order	**Mononegavirales**			
Family	Bornaviridae		Genus	Bornavirus
Family	Filoviridae		Genus	„Maburg-like viruses“ „Ebola-like viruses“
Family	Paramyxoviridae			
	Subfamily	Paramyxovirinae	Genus	Morbillivirus Respirovirus Rubulavirus
	Subfamily	Pneumovirinae	Genus	Pneumovirus Metapneumovirus
Family	Rhabdoviridae		Genus	Vesiculovirus Lyssavirus Ephemerovirus Novirhabdovirus Cytorhabdovirus Nucleorhabdovirus

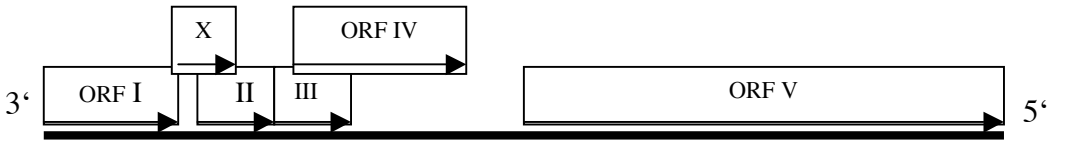
Table 1. Taxonomic structure of the order

There are four known virus strains: Borna V, Borna HE/80, Borna No/98, Borna H1766. The genomes of these strains have been sequenced and published; strain H1766 is almost 98% and 95% identical to strains V and HE/80, respectively, whereas strain No/98 is about 81% identical to the reference strains. These findings show that BDV is characterised by extraordinary sequence conservation uncommon for RNA

viruses [14,15,22,23]. Other BDV variants might be present outside the endemic regions and Staeheli et al. (2000) also suggest that a great number of strains may exist worldwide but the currently available detection methods only detect a fraction of them [24].

2.2.2. Genomic organisation

BDV is an enveloped, non-segmented, single- and negative-stranded RNA virus (NNS RNA). Replication and transcription of the BDV genome occur in the cell nucleus. While nuclear replication and transcription are found in segmented negative-strand RNA viruses and nucleo-rhabdoviruses (NNS RNA viruses of plants), BDV is the only known NNS RNA virus of animals with this property [25].



ORF I	Nucleoprotein (N)	p38/40
ORF II	Phosphoprotein (P)	p24
ORF III	Matrixprotein (M)	p16
ORF IV	Glycoprotein (G)	p57
ORF V	RNA Ploymerase (L)	p190
ORF X	(X)	p10

Fig. 1. 6 Open Reading Frames (ORF)

The genome size is about 8.9 kilo bases, which makes it the smallest among known negative-stranded RNA viruses. The organisation of the six major ORFs (see fig. 1) is similar to that of other Mononegaviruses [26]. Based on their positions in the viral genome (3’-N-X-M-G-L-5’) and the features of the corresponding amino acid sequences, these polypeptides are counterparts of the nucleoprotein (N), phosphoprotein (P), matrix protein (M), surface glycoprotein (G) and polymerase (L), respectively, which are also found in other negative-stranded RNA viruses [27]. Negative-stranded RNA viruses initiate infection by introducing their genetic material in the form of ribonucleoprotein complexes into the host cells. These complexes, which consist of the single-stranded RNA genome associated with the RNA-binding protein N, the polymerase cofactor P and the RNA-dependent RNA polymerase L, are transcriptionally active and direct the synthesis of viral mRNAs [28]. After production of sufficient amounts of newly synthesised viral proteins, replication is initiated and new

nucleocapsid can be formed. The major components of the nucleocapsid are N and P, which are both present in various isoforms, as a result of alternative splicing and internal translation initiation mechanisms. These mechanisms are also producing a number of isoforms of the other nucleocapsid components. One reason for this variety appears to be a mean for differential transcriptional activity. Specifically, it has been shown that both the composition and stoichiometry of nucleocapsid components are involved in the regulation of the BDV multiplication cycle [29-32]. Based on these distinct genetic features among known negative-stranded RNA viruses, BDV is considered to be the prototypic member of a new virus family, *Bornaviridae*, within the order *Mononegavirales*.

2.3. Hosts and Epidemiology

BDV infections mainly affect horses and sheep but the disease is not strictly limited to these two hosts, although the incidence in other animals appears to be very low. BDV was found in donkeys [33-37], goats [37] and cattle [38,39] with neurological disease and prominent lymphocytic infiltrations of the CNS. Recently, BDV antigen and RNA have been identified in the brain of two dogs with severe neurological disease that had lived in Austria and Japan, respectively [40,41]. Furthermore there is ongoing controversy whether BDV induces "staggering disease" in cats [42-47]. In a study from Switzerland, one of 180 brains of cats with histologically confirmed CNS disease showed evidence of BDV infection by immunohistochemistry [48]. The other 179 cat brains were negative for BDV by immunohistochemistry and RT-PCR [49]. In addition, the presence of BDV-specific RNA was demonstrated by *in situ* hybridisation in neurons of the cerebral cortex of a cat with paralytic disease, but without CNS inflammation [50]. Altogether, these data strongly suggest, that although natural BDV infections of cats may occur occasionally, BDV is not likely to be the aetiological agent of "staggering disease". However, the identification of BDV in hosts such as lynx [51], rabbits [52] and even ostriches [53,54] indicates that the virus has a broad host repertoire of various birds and mammals. Finally, experimental infection with BDV has been demonstrated in many laboratory animals, such as rats, mice, rabbits and gerbils.

As already mentioned, Borna Disease received its name from an equine outbreak in 1895/96 near the town Borna in Germany. More sporadic occurrence of BD had since been described in different areas of Germany [55-57]. The endemic area

also includes parts of the upper Rhine valley between Switzerland, Austria and the Principality of Liechtenstein [37,58]. The Bündner Herrschaft, Domleschg, Schanfigg and the St. Galler Rheintal are the mainly affected areas in Switzerland. Although BDV infections have been reported from northern Europe, USA, Japan, Iran and Israel [59] [60,61], clinical cases of horses and sheep have rarely been reported outside the endemic area [62]. On the other hand, sero-epizootiological studies have shown that BDV is geographically more widely distributed and is also present at higher rates in animals than previously thought. It remains unclear whether this is due to a larger dissemination of BDV or merely to a higher interest for the virus, associated with an improvement in the diagnostic methods [63,64].

However, many fundamental questions regarding the epidemiology of BDV remain unsolved. Most importantly, the way of transmission and the involvement of potential vectors remain elusive. BDV infectivity and RNA have been detected in body secretions, suggesting that BDV could be transmitted through salival, nasal, or conjunctival secretions, and particularly urine and faeces [65]. Animals become infected by direct contact with these secretions or by exposure to contaminated food or water. Since direct transmission of BDV from diseased horses or sheep to uninfected animals has not been proven, a different natural reservoir of BDV, which has not yet been identified, must exist. Several studies suggest that rodents could be vectors. Specifically, the urine of experimentally infected rats showed high BDV titers, and inoculation of adult black hooded rats with BDV has been shown to induce persistent infection in the absence of disease [66]. It has also been shown that BDV in fresh urine samples of carrier rats entered the brains of recipient rats via the olfactory route and can cause BD. Thus, susceptible farm animals possibly acquire the virus from persistently infected rats [67]. In addition, there is evidence for horizontal transmission via urine in the case of Lassa and Hanta virus. Interestingly recent RT-PCR and *in situ* hybridisation data of BDV in a fetus from a horse with BDV infection suggests intrauterine, i.e., vertical, transmission in nature [68]. There is, however, also the possibility that BDV is transmitted via arthropod vectors. Ticks, for example, were already discussed as a possible vector for BDV. They are obligate, blood-sucking members of the order *Ascarina* and class *Arachnida* and are very common agents of vector-borne diseases; e.g., they play an important role in transmitting viruses, bacteria, spirochetes, parasites and rickettsia. Ticks are divided into three families,

only two of them are capable of causing infection: soft ticks (*Argasidae*) and hard ticks (*Ixodidae*), the latter being responsible for most tick-related diseases. *Ixodes ricinus* is one of the most common hard ticks in Europe and can therefore be found in the endemic area as well. The life cycle of ticks stretches over two years and includes egg, larva, nymph, and adult. All stages except egg require a blood meal for morphogenesis. Lyme disease, Ehrlichiosis, Babesiosis or FSME are some examples of tick-borne diseases [69]. The idea, that ticks could be a vector for BDV is supported by the fact that BD tends to occur in spring and early summer and is more frequent in some years than in others. Moreover, ticks have been associated with transmission of an equine encephalomyelitis similar to BD in the Near East [70]. But BDV has never been isolated from insects in Europe so far and it is still controversial whether BDV can cause a viraemia, which would be required for any tick-borne infection.

BDV has gained major interest in 1985, when Rott et al. (1985) suggested that BDV or a BDV-like virus might be associated with various human psychiatric diseases [71], and since then the epidemiology of BDV was investigated intensively by medical and veterinary virologists. Antibodies recognising BDV specific antigens were found in the sera of some psychiatric patients with higher prevalence than in healthy controls. Accumulating evidence, using various serological techniques, confirmed the original findings and indicated that BDV infections in humans occurred worldwide and seemed to be associated with certain human mental diseases [72-83]. In general, the highest percentage of seropositive patients could be found in areas of Germany. Investigation of cerebral spinal fluid (CSF) from BDV-seropositive patients with acute psychiatric episodes (mainly schizophrenia and affective psychoses) showed intrathecally synthesised BDV-specific antibodies in 25% of patients [84]. However, solid proof for a role of BDV in human psychiatric disorders does not exist. There are several reports about the isolation of BDV RNA from peripheral blood or brain tissue of human psychiatric patients [85-88]. These reports, however, are not totally convincing and the human origin of most "human" BDV isolates reported so far was recently questioned by Schwemmle et al. (1999). They showed that the genomes of such "human" BDV isolates were closely related to BDV strains frequently used for experiments in the various reporting laboratories [89]. In conclusion, the question as to whether a potential BDV infection of humans can induce neuropsychiatric disease and thus whether BD can be called a zoonosis, is still controversial. Certainly all studies

published so far have reported a significantly higher prevalence of BDV-specific antibodies in sera and, if available, in CSF of neuropsychiatric patients when compared to healthy humans.

2.4. Clinical manifestations

Horses and sheep with BD exhibit a variety of clinical symptoms, predominantly behavioural abnormalities, apathy and movement disorders, which are not specific for BD but may also be seen in animals infected with other microorganisms invading the CNS.

The incubation period is variable, between two weeks and a few months. BDV infections in horses are often clinically inapparent, but they can result in simultaneous or consecutive disorders in behaviour, sensibility and motility. During the initial phase, nonspecific signs such as hyperthermia, anorexia, and alternance of colic and constipation are observed. During the acute phase, neurological signs result from meningoencephalitis, such as abnormal posture, ataxia, proprioceptive deficit and repetitive movements. These signs can be associated with abnormal reactions to external stimuli such as hyperexcitability, aggressiveness, lethargy, somnolence and stupor. In the final phase, paralysis can appear, followed by convulsions. Death usually occurs after one to three weeks and the death rate in horses is above 80%, and 50% in sheep [90,56,91]. In animals that have survived the acute phase of the disease, recurrent episodes may possibly appear for the rest of the animals life (chronic infection) with depression, apathy, somnolence, fearfulness, in particular after stress [64,90]. However, this is still controversial. Although the clinical pattern is still considered to represent classical BD, infection may also result in asymptomatic carrier status, subtle disturbances in learning and memory, profound disorders of behaviour and movement or death [24,92].

Various laboratory animals are susceptible to BDV infection. The incubation period, mortality and severity of the disease considerably depend on the infected animal species, viral variant and host immune status. In adult immunocompetent animals, the infection causes a meningoencephalitis as in horses and sheep. However, immunodepressed animals or animals with immature immune system show more discrete symptoms. Lewis rats for example are highly susceptible to the infection and represent an excellent model to study BD pathogenesis.

2.5. Immunopathology

The crucial importance of the immune system in BDV infection becomes obvious when athymic or immunocompromised animals are infected. In a detailed study the immunopathological basis of BD was established in rats [93]. It was shown, that an infection of neonate and immunosuppressed adult rats resulted in the absence of encephalitis and disease. The frenzied behaviour and subsequent blindness in immunocompetent adults were therefore attributed to a uniquely transient immunopathologic reaction targeted to centers in the limbic system and retinal neurons [93].

BDV infection is noncytolytic and persistent. Mechanisms of pathogenicity in the brain may include direct interaction with intracellular signaling and function, or interference with intracellular communication essential to brain function, probably through soluble factors such as cytokines, neurotrophins, and/or neurotransmitters [94,95].

The adult- and the neonatally infected Lewis rat models are the best-studied systems for BDV infection. Infection of adult Lewis rats produces a prominent neurobehavioral disorder and is characterised by pronounced immunopathology. In the acute phase (4 to 8 weeks post infection), cellular infiltrates, such as $CD4^+$ and $CD8^+$ T cells, natural killer cells and macrophages, and Th_1-type cytokines are prominent in perivascular and parenchymal regions of the CNS. In the chronic phase (15 weeks post infection and beyond), a decline in infiltrates is accompanied by an increase in Th_2-type cytokines and a shift to humoral immune response [96]. $CD8^+$ cells mediate destruction of virus-infected cells in the CNS, whereas $CD4^+$ T cells promote production of antiviral antibodies. Although antibodies to N and P generated during the acute phase of the disease are non-neutralising [97], antibodies with neutralising capacity increase dramatically after the acute phase [98] and likely participate in restriction of virus to neural tissue [99].

The neonatal rat model does not show overt immunopathology; instead, despite high virus load in the brain and lifelong persistence, animals infected within the first 12h of life develop a mild behavioural syndrome and restricted neuropathology that may provide a more intriguing model for neuropsychiatric disorders. Although cellular inflammatory response to BDV following neonatal infection is restricted, a phenomenon ascribed to the immaturity of rat postnatal

immune function, a brief surge in mononuclear cell infiltrates occurs [100,101], along with elevations in expression of proinflammatory cytokine [100,101], chemokine [102], and chemokine receptor transcripts[103,104].

In conclusion, BD in rats appears to be a $CD4^+$ T-cell-dependent immunopathological disease, in which $CD8^+$ T-cells and/or $CD8^+$ T-cell-mediated cytodestructive mechanisms are operative, leading to tissue destruction, organ atrophy, and clinically to organ dysfunction and disease [105]. The similarity of inflammation and the cellular composition of the infiltrates in ovine and equine BD lead to the assumption that BDV infection induces the same immunopathological process as in laboratory animals [106].

2.6. Diagnosis

Reliable *intra vitam* diagnosis of BD is still difficult. Horses and sheep with BD exhibit a variety of clinical symptoms, which may be unspecific for BD and can be caused by other CNS diseases. Increased protein content and mononuclear pleocytosis may be seen in cerebrospinal fluid (CSF) of animals with BD. However, these changes are not specific for BD but rather represent non-specific indicators of viral meningoencephalitis [57]. BDV specific antibodies in serum and / or CSF are better indicators. Indirect immunofluorescence assay (IFA) appears to be the most reliable method [34,107]. However, it was shown by Caplazi et al. (1999), that antibodies could not be found regularly in animals, which were diagnosed as BD by histology [37] and that the prevalence in healthy sheep of the endemic area (Switzerland, Principality of Liechtenstein) for slaughter was 6,4% [108,109]. Therefore post-mortem confirmation by histological analysis of brain tissue is required.

On a histological level, variable degrees of encephalitis are observed with BD [35,106,110]. It is characterised by disseminated mononuclear meningitis and polioencephalomyelitis with subsequent neuronal degeneration. Usually, a site predilection for areas of the limbic system, particularly the hippocampal formation can be noted, whereas the brain stem and cerebellum are relatively spared [106]. Traditionally, Joest-Degen inclusion bodies in nuclei of infected neurons have served as BDV specific markers [111], but they cannot consistently be seen by routine histology in brains of animals suffering from BD. $CD4^+$ T cells are predominantly present at

perivascular sites, whereas $CD8^+$ T cells are found both in the perivascular cuffs and in the brain parenchyma [57,106].

Immunohistological analysis of paraffin-embedded brain sections with monoclonal antibodies against the major BDV antigens p40 and p24 consistently showed that virus-infected cells are non-uniformly distributed in brains of diseased animals and that antigen-positive neurons are found most frequently in the hippocampus [35,36,106].

To improve the sensitivity of BDV antigen detection, an antigen-capture ELISA was introduced which uses a cocktail of monoclonal antibodies and monospecific polyclonal antiserum for antigen capture and detection, respectively [34]. Later, specific antibodies against BDV-p40 and –p24 antigens were developed and a simple, quantitative and sensitive antigen capture ELISA system was established [29].

RT-PCR or RT-nested PCR analysis of native or formalin fixed brain tissue is a sensitive alternative technique that may be used to confirm the clinical diagnosis of BD [33,36,57,112-115]. However, since high-sensitivity PCR technology is prone to contamination artefacts, confirmatory laboratory diagnosis which relies exclusively on RT-nested PCR results should not be considered definitive [24].

A possibly more reliable, though less sensitive method to detect viral transcripts is the analysis of thin sections of paraffin-embedded brains by *in situ* hybridisation using RNA probes complementary to the major transcripts of BDV [35,57,111,116,24].

The aim of the present study was to establish a more sensitive and reproducible method for the detection of BDV from various tissues, which also allows for quantitative comparison of virus load. We therefore used TaqMan® Real Time RT-PCR as the method of choice. In addition we wanted to validate this method by screening brain tissue samples from sheep and horses, which had been diagnosed as BD by histological and immunohistochemical examinations. Finally, we investigated the potential role of a blood sucking tick species, *Ixodes ricinus*, as a possible vector for the transmission of BDV.

3. Materials and Methods

3.1. Primers for Real Time RT-PCR (TaqMan®)

For the amplification of BDV cDNA, consensus primers and an internal oligonucleotide as a probe were designed based on the open reading frames of *p40* and *p24* from the four known BDV virus strains Borna V, Borna HE/80, Borna No/98 and Borna H1766 (for alignments of p40 and p24, see results section 4.1.1. Consensus primers).

Primers and probes used for the amplification of BDV cDNA:

p40For: 5' GGTTTAAAACTATGATGGCAGCCTTA 3'
p40Rev: 5' GTGGATTAAACATCTGGAGTAGTGTAGC 3'
p40 Probe: 5' ACCGGCCATCCCATGGTGAGAC 3'

p24For: 5' TCCCTGGAGGACGAAGAAGAT 3'
p24Rev: 5' CTTCCGTGGTCTTGGTGACC 3'
p24 Probe: 5' CCAGACACTACGACGGGAACGA3'

The internal probe was labelled at the 5' end with the reporter dye FAM (6-carboxyfluorescein) and at the 3' end with the quencher TAMRA (6-carboxytetramethylrhodamine). Furthermore, nuclease oligoprobes were designed according to the following requirements, (i) a length of 20 to 40 nt, (ii) a GC content of 40-60%, (iii) no runs of a single nucleotide, particularly G, (iv) no repeated sequence motifs, (v) an absence of hybridisation or overlap with the forward or reverse primers and (vi) a T_M at least 5°C higher than that of the primers, to ensure that the oligoprobe has bound to the template before extension of the primers can occur [117].

Amplification with p40 primers yields a product of 78bp and with p24 primers of 69bp, respectively. Primers and probes were synthesised by Applied Biosystems, Weiterstadt, Germany.

As an endogenous control, eukaryotic 18s ribosomal RNA (Applied Biosystems, Foster City, CA, USA) was used. The probe was labeled with VIC™ dye-MGB.

3.2. Plasmids

The two plasmids pI_pPG401:p40-dHLX-ABP-6xHis and pI_pCRII-ORFI (both kindly provided by Rolf Kocherhans, Institute of Virology, Vetsuisse-Faculty, University of Zurich) containing the *p40* ORF were used to determine the molecular sensitivity of the system (see section 3.9. Sensitivity and Specificity).

3.3. Cell culture

MDCK cells were used to determine the cellular sensitivity of the system (see section 3.8. Sensitivity and Specifcity) and for experimental infection of ticks. Cells (ATCC Nr. CCL-34) were grown in Iscove's Mod. Dulbeco's Medium (Sigma chemical co., St. Louis, USA). To 500ml medium 50ml 10% fetal bovine serum, 12,5ml hepes buffer (Sigma chemical co., St. Louis, USA, 1M, H0887), 5ml L-Glutamine (Sigma chemical co., St. Louis, USA, 200mM, G7513) and 5ml Penicillin-Streptomycin solution (Sigma chemical co., St. Louis, USA, P0781) were added. The cells were trypsinised (Trypsin-EDTA solution, 1x, Sigma chemical co., St. Louis, USA, T3929) and diluted twice each week and maintained at 37°C with 5% CO_2. For RNA isolation, cells were stained with trypan blue, counted in a Neubauer chamber and cell numbers normalised. BDV-infected MDCK cells (BDV isolate of the horse H1766) were a kind gift by Dr. S. Herzog, Institute of Virology, University of Giessen, Germany.

3.4. Tissue samples

Ovine and equine brain samples included in this study were collected during autopsies at the Institute of Veterinary Pathology, Vetsuisse-Faculty, University of Zurich from the years 1996 to 2003. The brain was removed from the cranium and divided in two halves by longitudinal section. From one halve of the brain, tissue samples (approx. 1 cm^3) were collected with a scalpel, stored in small petri dishes at –20°C or –70°C, respectively, until further use. The other halve of the brain was fixed by immersion in 4% buffered formaldehyde for at least 48 hours and then sliced according to five standard cuts of the brain. Sections were stained in haemalaun-eosin (HE) for microscopical examination to estimate the inflammatory reaction characterised by

disseminated mononuclear meningitis and polioencephalomyelitis with subsequent neuronal degeneration [106].

Specifically, samples from the cortex, hippocampus and cerebellum (or lobus piriformis, if no cerebellum available) were collected from a total of 6 BDV-infected animals (three sheep and three horses). As a negative control, brain samples from two uninfected animals (one sheep and one horse) were collected.

Sheep samples:

a) BDV infected	S03-0057	(Cortex, Hippocampus, Cerebellum)
	S02-1736	(Cortex, Hippocampus, Cerebellum)
	S99-1356	(Cortex, Hippocampus, Cerebellum)
b) Non infected	S02-0857	(Cortex, Hippocampus, Cerebellum)

Horse samples:

a) BDV infected	S00-0913	(Cortex, Hippocampus, Cerebellum)
	S99-0598	(Cortex, Hippocampus, L. piriformis)
	S96-0868	(Cortex, Hippocampus, L. piriformis)
b) Non infected	S01-1038	(Cortex, Hippocampus, Cerebellum)

3.5. Immunohistochemical examination of tissue samples

Immunohistochemical examination of the brain samples was performed in parallel to the TaqMan® PCR with slides of the animals and brain sites described above. Formalin fixed and paraffin embedded sections were deparaffinized and rehydrated in xylene and alcohol. Sections were then counterstained with haemalaun for 2 min and watered for 3 to 5 min. After digestion with 0,1% Pronase (DAKO Cytomation, Copenhagen, Denmark, S2013) for 10 min at room temperature, endogenous peroxidase was blocked by incubation with H_2O_2 (3% in H_2O + 0,2% in NaN_3) for 10 min at room temperature. Sections were incubated with a monoclonal mouse-anti BDV antibody, which recognises p38/40 (L. Stitz, Tübingen, Germany*)* in a dilution of 1:10'000 over night at room temperature. Then sections were incubated for 10 min at room temperature with a secondary anti-mouse/anti-rabbit-biotinylated antibody (ChemMate™-KIT, DAKO Cytomation, Copenhagen, Denmark, K5003). Finally, streptavidin-peroxidase (ChemMate™-KIT, DAKO Cytomation, Copenhagen, Denmark, K5003) was added for 10 min at room temperature. After each step slides were thoroughly washed with PBS (Phosphate buffer solution, pH 8.0). Finally, the

reaction was visualised with AEC substrate (AEC-Substrate chromogen kit, Zymed, San Francisco, CA, USA, 00-2007).

3.6. Experimental infection of ticks

Adult female ticks (*Ixodes ricinus)* were inoculated with BDV-infected or non-infected MDCK cells according to the method of Burgdorfer [118]. For experimental infections, a suspension of 2 x 10^6 cells/ml (approx. 75% of those infected with BDV) was used. Individual animals were fixed dorsally on adhesive foil and then a capillary tube containing the suspension of BDV-infected MDCK cells was pushed over the hypostome and cheliceral elements, excluding the pair of palpi. Blocks of plasticine were used to hold the capillary in position. The whole procedure was performed with the aid of low power magnification. The ticks were fed at room temperature for 1 to 3 hours, during which period they ingested 0.01 to 0.03 ml of the cell suspension (i.e., 2-6 x 10^4 cells), which is the amount they normally take up according to Burgdorfer et al. [118]. After a waiting period of one to three hours, most of the ticks got thicker and defecation was admitted as evidence of the absorption. The partially fed ticks, 80 to 100 ticks at a time, were removed and stored in tubes with a perforated cover to ensure air supply. The tubes itself were kept in a small box which was lined with wet gauze. Since it is critical to keep the animals at humid climate, blades of grass were added in addition.

Fig. 2. Artificial feeding of ticks

3.7. RNA extraction and reverse transcription

Total RNA from MDCK cells and vaccine suspension (see section 3.9.1. Specificity assay) was directly extracted with RNeasy Mini Kit (QIAGEN AG Basel, Switzerland). The other samples were treated as follows before RNA extraction. Brain tissue samples (approx. 30 mg/sample) were disrupted and homogenised with QIAshredder and total RNA subsequently isolated with RNeasy Mini Kit. Ticks were disrupted and homogenised with a mortar in 400µl RLT buffer, lysis buffer provided with the RNeasy Mini Kit containing 10µl β-Mercaptoethanol per ml buffer, prior to RNA isolation with RNeasy Mini Kit. RNA from all samples was then digested with 1µl RNase-free DNase (Promega, Dubendorf, Switzerland) for 1h at 37°C, followed by a deactivation step at 95°C for 5 min.

Isolated and purified RNA was reverse transcribed into cDNA using the Reverse Transcriptase System (Promega, Dubendorf, Switzerland). Briefly, total RNA was incubated with random primer and 30 units RT for 1h at 42°C. Then the RT was inactivated for 5min at 95°C. The cDNA was stored at –20° C until further use.

3.8. PCR amplification by TaqMan® technology

PCR amplification and quantification were performed on an ABI 7700 real-time Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland).

3.8.1. Principles

The basis for PCR quantitation in the ABI 7700 system is to continuously measure PCR product accumulation using a dual-labeled fluorogenic oligonucleotide probe called a TaqMan® probe. This probe is composed of a short (ca. 20-25 bases) oligodeoxynucleotide that is labeled with two different fluorescent dyes. On the 5' terminus is a reporter dye (FAM) and on the 3' terminus is a quenching dye (TAMRA). This oligonucleotide probe sequence is homologous to an internal target sequence present in the PCR amplicon. When the probe is intact, energy transfer occurs between the two fluorophores and the quencher suppresses emission of the reporter. During the extension phase of PCR, the probe is cleaved by 5' nuclease of Taq polymerase thereby releasing the reporter from the oligonucleotide-quencher and producing an increase in reporter emission intensity (see fig. 3). The ABI Prism 7700 uses fiber optic systems, which connect to each well in a 96-well PCR tray format. The laser light source excites each well and a CCD camera measures the fluorescence spectrum and intensity from each well to generate real-time data during PCR amplification. The ABI 7700 Prism software examines the fluorescence intensity of reporter and quencher dyes and calculates the increase in normalised reporter emission intensity over the course of the amplification. The results are then plotted versus time, represented by cycle number, to produce a measure of PCR amplification. To provide precise quantification of initial target in each PCR reaction, the amplification plot is examined at a point during the early log phase of product accumulation. This is accomplished by assigning a fluorescence threshold above background and determining the time point at which each sample's amplification reaches the threshold (defined as the threshold cycle number or C_T). Differences in threshold cycle number are used to quantify the relative amount of PCR target contained within each tube as described previously.

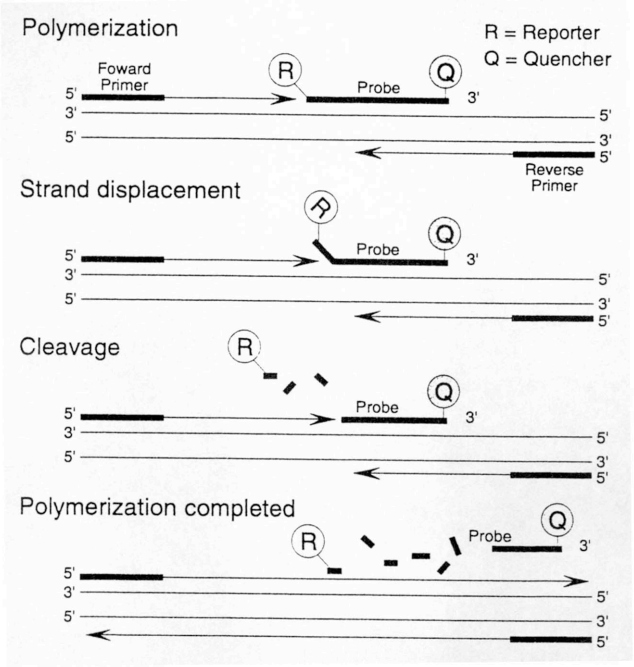


Fig 3. Scheme of the TaqMan® technology (TaqMan® Universal PCR Master Mix, Protocol, Applied Biosystems, Foster City, CA, USA)

3.8.2. Reaction conditions

PCR amplification was carried out in 25-µl reaction mixtures per well containing 12.5µl of 2x TaqMan® Universal PCR Master Mix (Applied Biosystems, foster City, CA, USA), 600nM of each primer, 80nM (p40-56T) and 160nM (p24-54T) of the probes, and 3,5µl of cDNA. In addition, 18s rRNA was amplified as an internal control to monitor the quality of the extracted RNA (PE Biosystems, Warrington, UK). The same 18s primers/probe were used for amplification of cDNA from MDCK cells, tissues and ticks. PCR conditions were set as follows: 2 min at 50°C to activate the Uracil-N-Glycosilase, which detects possible former PCR products and destroys them, and then 10 min at 95°C to inactivate the Uracil-N-Glycosilase and activate the polymerase followed by 40 cycles consisting of denaturation at 95°C for 15s and annealing and elongation at 60°C for 1 min. The data were analysed with the sequence detector software (version 1.7). Signals were regarded positive if the fluorescence intensity (increase of fluorescence ΔR_n) exceeded 10 times the standard deviation of the baseline fluorescence (threshold cycle [C_T]). C_T values of 40 were regarded as negative. The threshold cycle or C_T value is the cycle at which a statistically significant increase in ΔR_n is first detected.

3.9. Specificity and sensitivity assays

3.9.1. Specificity assay

To determine whether the p24 and p40 consensus primers were specifically amplifying cDNA from BDV, total RNA from other virus species (see table 2) was used as control template (see also 4.1.3. Specificity). Virus RNA was extracted from vaccine suspensions containing inactivated virus using RNeasy Mini Kit. As a positive control, brain tissue (cortex) of a sheep, dissected at the Institute of Veterinary Pathology, Vetsuisse-Faculty of the University of Zurich, and suffering from BD (S01-1629) was applied.

Order	**Mononegavirales**		
Family	Paramyxoviridae	Genus	Parainfluenzavirus 2[1)]
			Parainfluenzavirus 3[2)]
			Canine distemper virus[1)]
Family	Rhabdoviridae	Genus	Lyssavirus[3)]
Order	**Nidovirales**		
Family	Coronaviridae	Genus	Porcine Epidemic Diarrhoea virus [2)]

1) Total RNA from vaccine solution Vetamun Live Standard, Intervet International B. V., Boxmeer, NL
(In addition to theses two viruses, Canine Adenovirus, Canine Parvovirus, Leptospira interrogans, Serovar Canicola and Serovar Icterohaemorrhagiae are contained in the vaccine as well.)

2) Total RNA from PI3 and PEDV infected cells, kind gift of the Institute of Virology, Vetsuisse-Faculty, University of Zurich

3) Total RNA from vaccine solution Rabdomun, ESSEX, Tierarznei, Germany

Table 2. Templates used to determine the primer specificity for BDV detection

3.9.2. Sensitivity assay

3.9.2.1. Molecular sensitivity

To determine the molecular sensitivity of the system, plasmids containing the p40 ORF (for details see section 3.2) were serially diluted by 10-fold dilution steps

covering a range between 10^{8} and 10^{-2} plasmid molecules per PCR reaction. PCR conditions were as described in 3.7.2.

3.9.2.2. Cellular sensitivity

To determine the cellular sensitivity of the system, BDV-infected MDCK cells (and as a control uninfected cells) were serially diluted and RNA extracted for TaqMan® PCR (for cell culture and RNA extraction see sections 3.3 and 3.7, respectively). Briefly, cell numbers of BDV-infected and non-infected cells were normalised by addition of PBS. Then, BDV-infected cells were diluted with non-infected cells by 10-fold dilution steps. The tubes were centrifuged and RNA was extracted from the pellet as described above. Cell numbers were also monitored by amplification and quantification of cell-specific RNA (with 18s rRNA primers, see above).
All the following calculations were performed according to the relative standard curve method.

4. Results

4.1. Establishment of the Real Time RT-PCR (TaqMan®)

4.1.1. Consensus primers

The negative-stranded RNA genome of BDV contains 6 ORFs. The two major gene products are the nucleoprotein p40 and the phosphoprotein p24. We designed primer and probe sequences based on the consensus sequences of the four major BDV strains Borna V, Borna HE/80, Borna H1766 and Borna No/98.

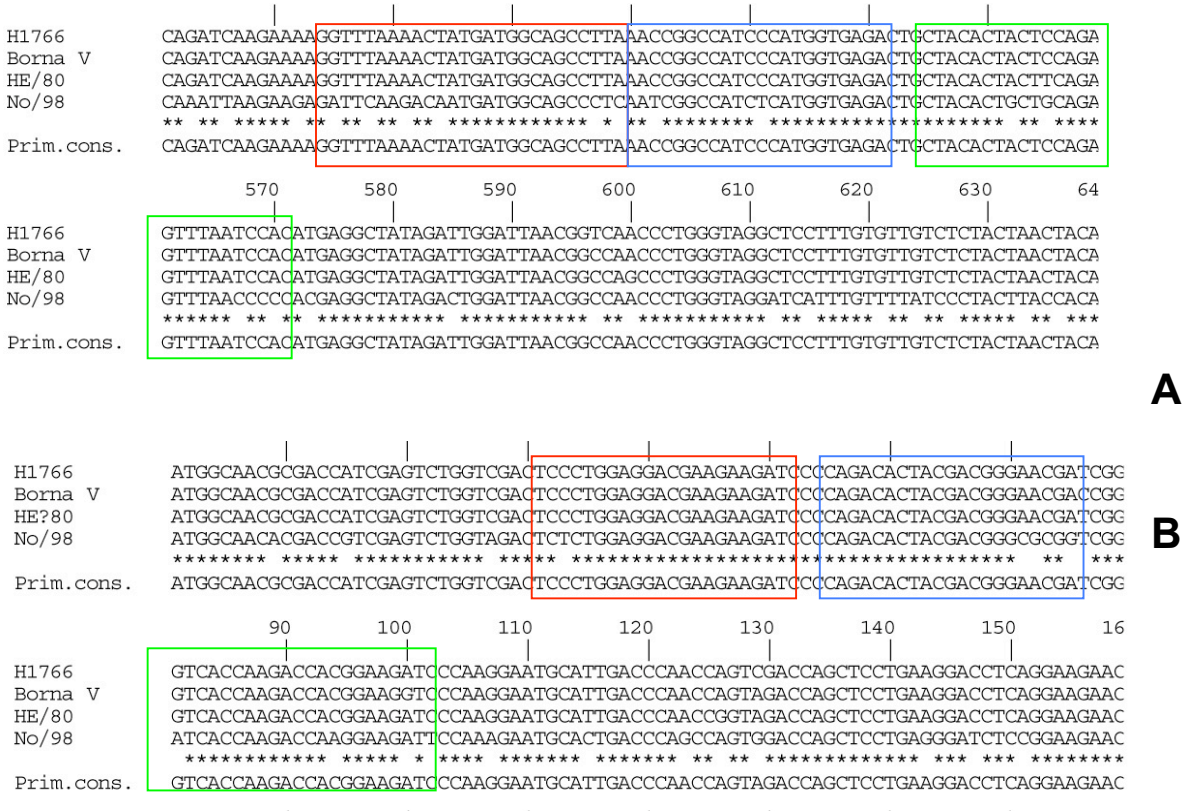


Fig. 4. Partial sequence alignment of the genomic RNA for p40 (A) and p24 (B) of the four BDV strains. The primary sequence consensus is shown at the bottom of the alignment. Asterisks represent identical positions. Red box: Forward consensus. Blue box: Probe consensus. Green box: Reverse consensus.

Expected *p40* PCR product:

5'-
GGTTTAAAACTATGATGGCAGCCTTAAACCGGCCATCCCATGGTGAGACT
GCTACACTACTCCAGATGTTTAATCCAC-3'

Expected *p24* PCR product:

5'-
TCCCTGGAGGACGAAGAAGATCCCCAGACACTACGACGGGAACGACGGG
TCACCAAGACCACGGAAG-3'

To rule out unspecific amplification of host cDNA, the expected PCR product was used as a query sequence in a BLAST search of the non-redundant nucleotide sequences deposited at GenBank (*www.ncbi.nlm.nih.gov*). This search revealed no significant match apart from BDV for both sequences.

4.1.2. Optimisation of the Real Time RT-PCR

In an initial experiment, three different primer concentrations (5pmol/µl, 2pmol/µl and 0,5pmol/µl) and probe dilutions (0,33pmol/µl, 0,66pmol/µl and 1,33pmol/µl) were tested to optimise the Real Time RT-PCR reaction. As a target, brain tissue samples (hippocampus) of a BDV infected sheep was used. Ideally, the slope in the linear phase of increase in fluorescence should be approx. 2 [3.1], reach a plateau at least at 1 and the C_T value should be as small as possible. These criteria were best met at primer concentrations of 5pmol/µl, and probe concentrations of 0,66pmol/µl for p40 and 1,33pmol/µl for p24. Accordingly, the threshold for the increase in fluorescence over time (or cycles), ΔRn, was set as 10^{-2}.

4.1.3. Specificity

To determine the specificity of the two primer pairs for the detection of BDV, total RNA from vaccine suspensions and infected cells as specified in section 3.9.1. Specificity assay, table 1, was reverse transcribed and used as a template for TaqMan® PCR. The results using primers and probe for p40 are shown in fig. 5. Primers and probe for p24 gave similar results but the data are not shown. The C_T values (the cycle number at which ΔRn meets 10^{-2}) for all RNA viruses tested (four from the order Mononegavirales, one Nidovirales) were 40 and therefore negative. In contrast,

reverse transcribed extracted total RNA from a control brain sample (cortex) of a sheep suffering from BDV yielded a positive C_T value of 17 cycles. The designed primers were therefore regarded suitable for the specific detection of BDV.

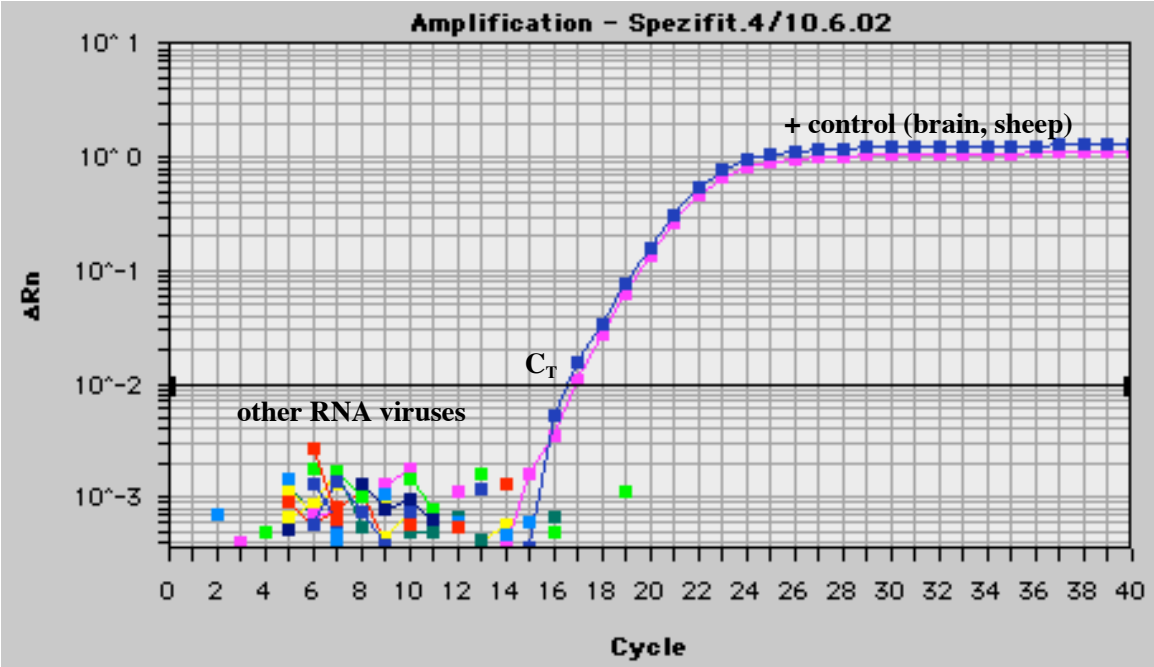


Fig. 5. Specificity of p40 primers and probe for BDV detection. Extracted RNA from brain tissue (BDV positive sheep) or vaccine suspensions (other RNA viruses) was reverse transcribed and used as a template for TaqMan® PCR. Colored squares (under the treshold): PI2 (Parainfluenzavirus 2), PI3 (Parainfluenzavirus 3), CDV (Canine distemper virus), Lyssavirus, PEDV (Porcine Epidemic Diarrhea virus). Blue and purple squares (curve): amplification from control sheep; p40 primers yields a positive C_T value of 17.

4.1.4. Sensitivity

4.1.4.1. Molecular sensitivity

In order to establish how many DNA molecules could be detected with Real Time RT-PCR (molecular sensitivity), a 10-fold dilution series (covering the range between 10^8 and 10^{-2} plasmid molecules per reaction) of the plasmids pI_pPG401:p40-dHLX-ABP-6xHis and pI_pCRII-ORFI, both containing the *p40* ORF, was tested. In four independent experiments, with Real Time RT-PCR we were able to detect 10 or more molecules per reaction (see figs. 6 and 7). In single experiments it was even possible to detect less than 10 molecules. It was concluded that the molecular sensitivity ranged between the detection of 1 and 10 molecules and therefore the assay proved to be highly sensitive.

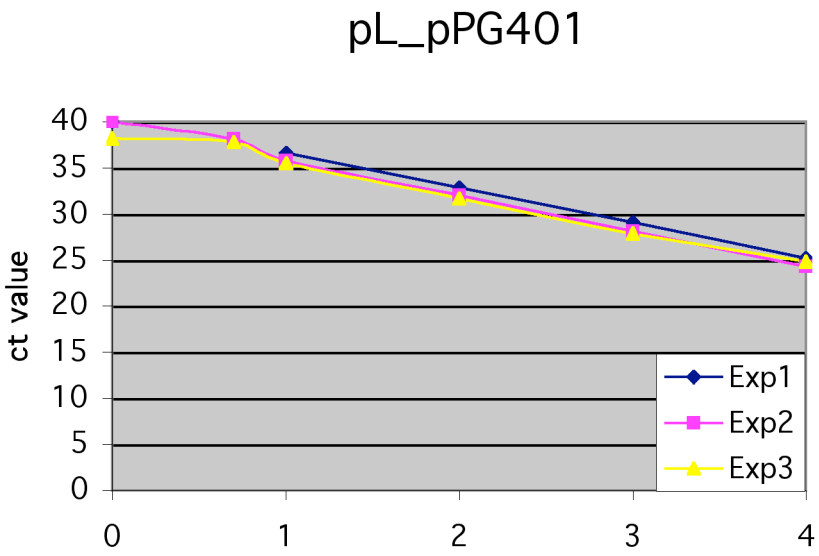


Fig. 6. Molecular sensitivity as determined with a dilution series of plasmid pL_pPG401 and primers p40. The higher the C_T values, the lower the virus load. x-axis: log plasmid number. y-axis: corresponding C_T value.

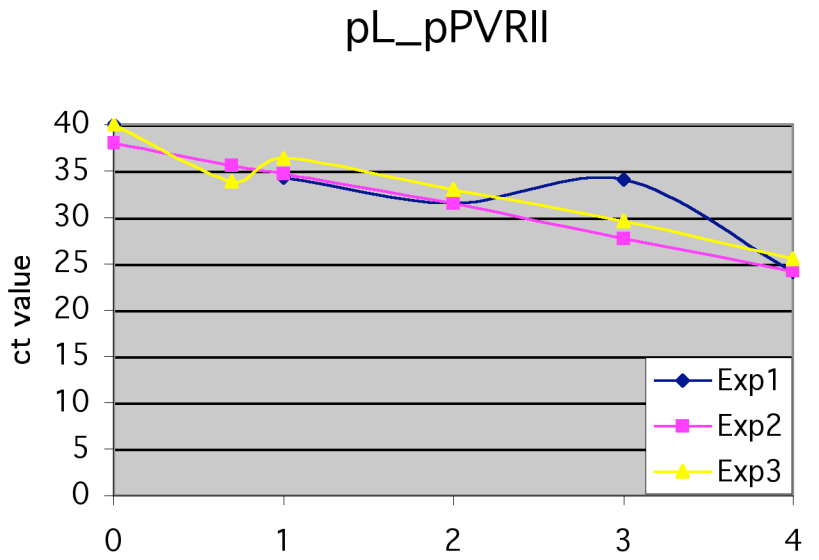


Fig. 7. Molecular sensitivity as determined with a dilution series of plasmid pL_pPVRII and primers p40. The higher the C_T values, the lower the virus load. x-axis: log plasmid number. y-axis: corresponding C_T value.

4.1.4.2. Generation of relative standard curves using BDV infected MDCK cells

As a prerequisite for the application of a BDV-specific Real Time RT-PCR, we chose to use the relative standard curve method to compare different samples on a semi-quantitative level. MDCK cells persistently infected with BDV were used for the generation of relative standard curves for p40 and p24, and 18s rRNA as an

endogenous control. Normalised infected MDCK cells were serially diluted in 10-fold steps. From these cells, RNA was extracted and reverse transcribed as described above. The TaqMan® results from the serial dilutions were then used to generate relative standard curves (y = ax+b) for the virus RNA using p40 and p24 primers, and for the host cell RNA (i.e., the total RNA used for the reverse transcription reaction) using 18s rRNA primers and probe. The virus-specific standard curves (see figs. 8 and 9) were used as basis to normalise the signals (C_T values) from p24 and p40 primer pairs from all experimental data and account for differences in the kinetics of the PCR reactions between the two primers (represented by differences in the slope a). A third standard curve representing the signal from 18s rRNA primers in a dilution series of uninfected MDCK cells (fig.10) was then used as an endogenous control to calibrate the system (hence termed the calibrator) against differences in the efficiency of the RNA extraction or cDNA synthesis of single experiments.

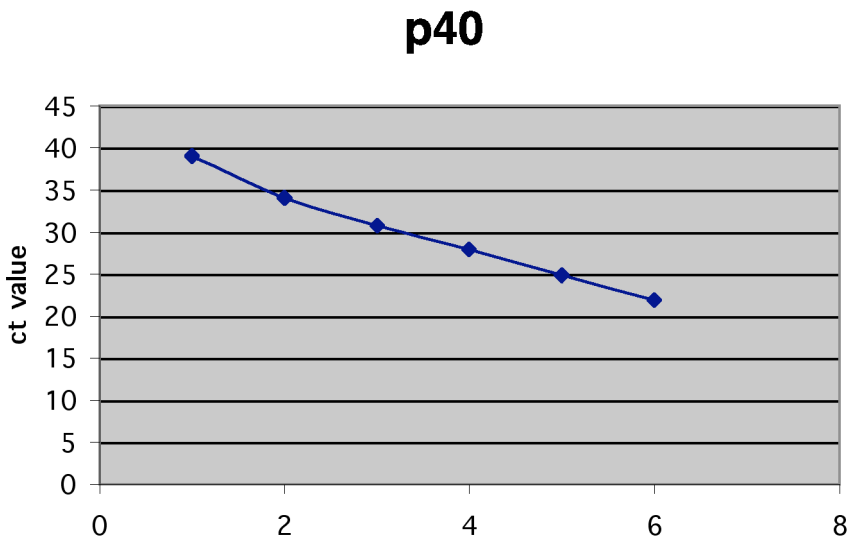


Fig 8. Relative standard curve (y= ax + b) for virus load in infected MDCK cells using p40 primers. The slope (a) amounts to –3.3317. (y = -3.3317x + 41.111). x-axis: log number BDV infected MDCK cells. y-axis: corresponding C_T value. The higher the C_T values, the lower the virus load.

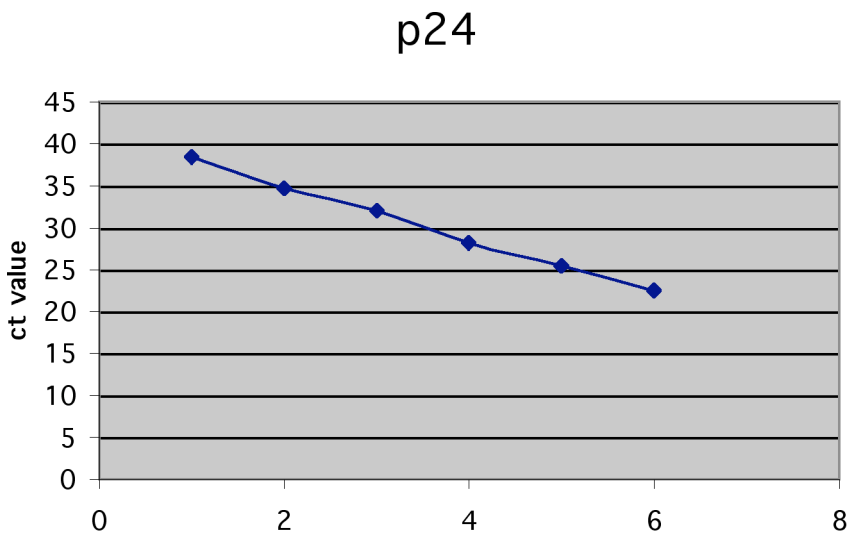


Fig 9. Relative standard curve (y= ax + b) for virus load in infected MDCK cells using p24 primers. The slope (a) amounts to –3.1796. (y = -3.1796x + 41.369). x-axis: log number BDV infected MDCK cells. y-axis: corresponding C_T value. The higher the C_T values, the lower the virus load.

For all experimental samples (sheep, horse, ticks), the target quantity (p40, p24 values) was determined from the corresponding standard curve and divided by the target quantity of the calibrator (18s rRNA). Therefore, the calibrator becomes the 1x sample, and all other quantities are expressed as an n-fold difference relative to the calibrator.

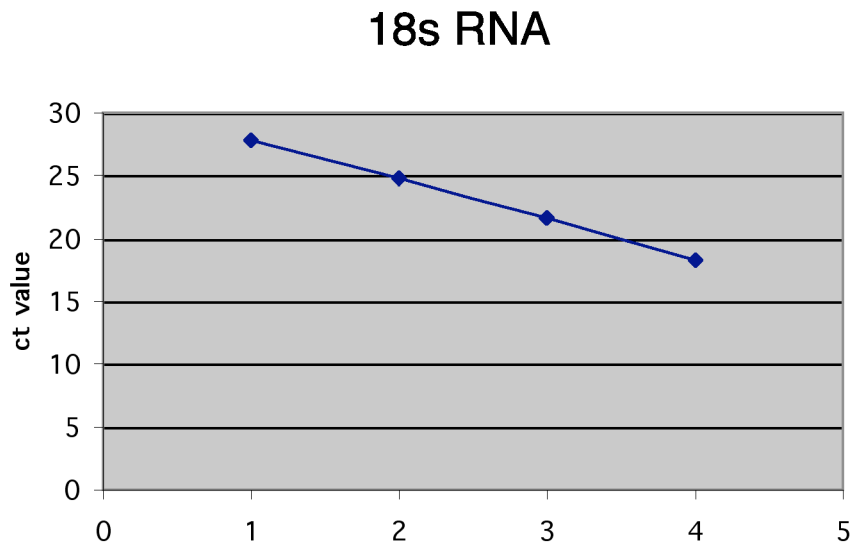


Fig. 10. Relative standard curve (y= ax + b) for total RNA as represented by the amount of 18s rRNA in a dilution series of uninfected MDCK cells. The slope (a) amounts to –3.1775. (y = -3.1775x + 31.063). x-axis: log plasmid number. y-axis: corresponding C_T value. The higher the C_T values, the lower the virus load.

4.2. Application of the Real Time RT-PCR

4.2.1. Cellular sensitivity in persistently BDV infected MDCK cells

After generating relative standard curves for p40, p24 and 18s rRNA, we tested the cellular sensitivity of the Real Time RT-PCR to detect BDV in the persistently infected MDCK cells. BDV-infected MDCK cells were diluted in 10-fold dilution steps and complemented with non-infected MDCK cells to obtain a uniform cell number for RNA extraction of 10^6 cells. As a negative control (threshold, see fig. 11), RNA from non-infected MDCK cells was used as a template As shown in fig.11, it was consistently possible to detect one single infected cell in 1 million uninfected cells, i.e., the cellular sensitivity of the Real Time RT-PCR was between 0.1 and 1.

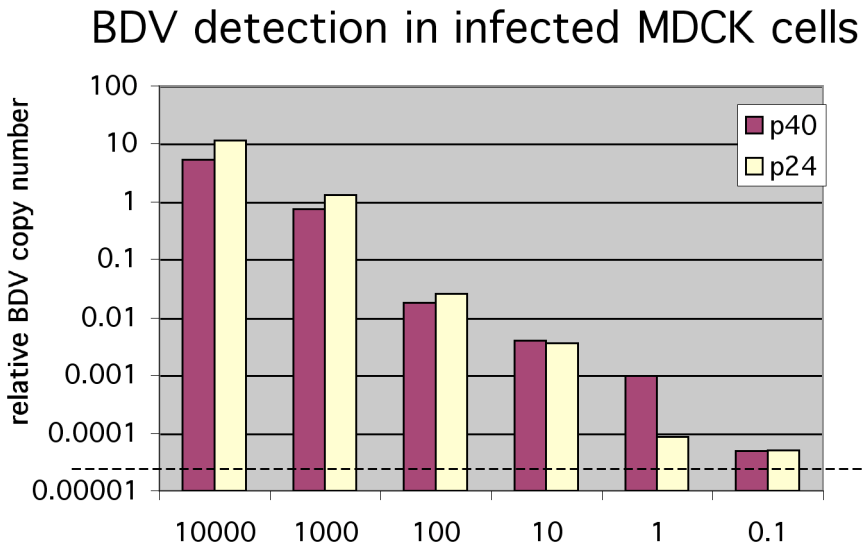


Fig. 11. Distribution of virus load in a dilution series of MDCK cells persistently infected with BDV. The threshold for both primers is 0.00004 (dashed line). Bars represent the average of 2 independent experiments. x-axis: number infected MDCK cells. y-axis: relative BDV copy number.

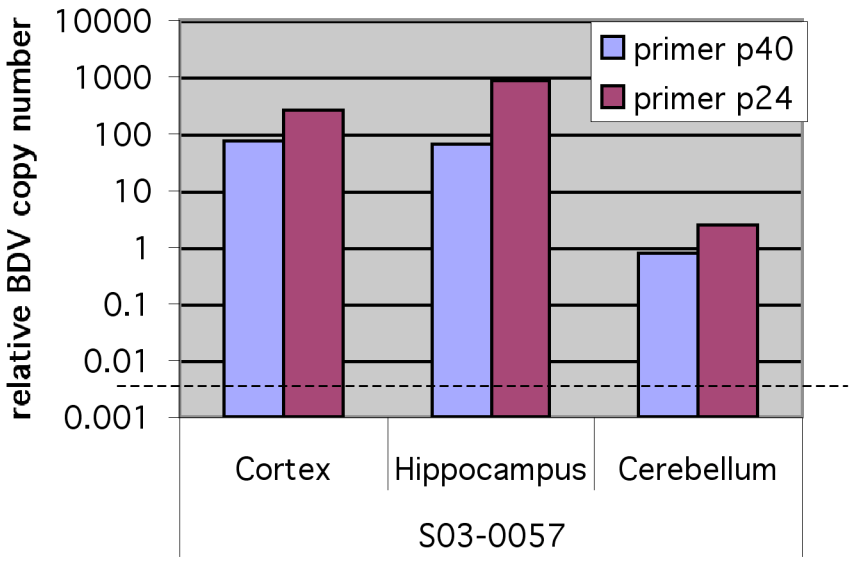
4.2.2. Brain tissues from BDV infected sheep and horses

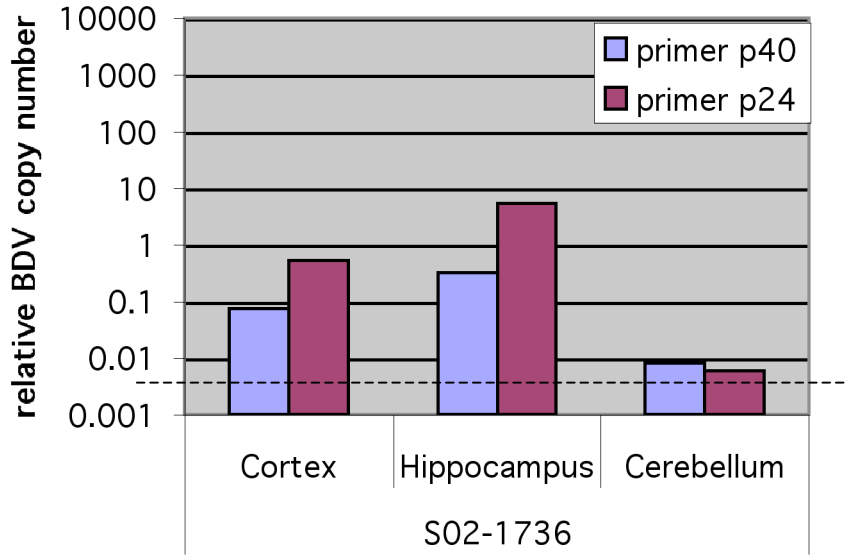
Frozen brain samples from three BDV-positive sheep and horses, respectively, were processed as described in 3.4 and analysed with TaqMan® PCR. The negative threshold was generated from brain tissue samples from a non infected sheep and horse (see figs. 12 and 14 and table 3). Total RNA from each tissue was extracted in three independent experiments, and the corresponding cDNA run twice on the ABI

Prism 7700. Finally, the C_T values were processed using the relative standard curves for p40, p24 and 18s rRNA, generated with infected (p40, p24 and 18s rRNA) MDCK cells.

4.2.2.1. Sheep

Since the three sheep chosen for examination were diagnosed positive according to histological and immunohistochemical data, it could be expected that the more sensitive Real Time RT-PCR method would detect the virus as well. Indeed, all three sheep showed presence of viral RNA in three independent brain sections measured.





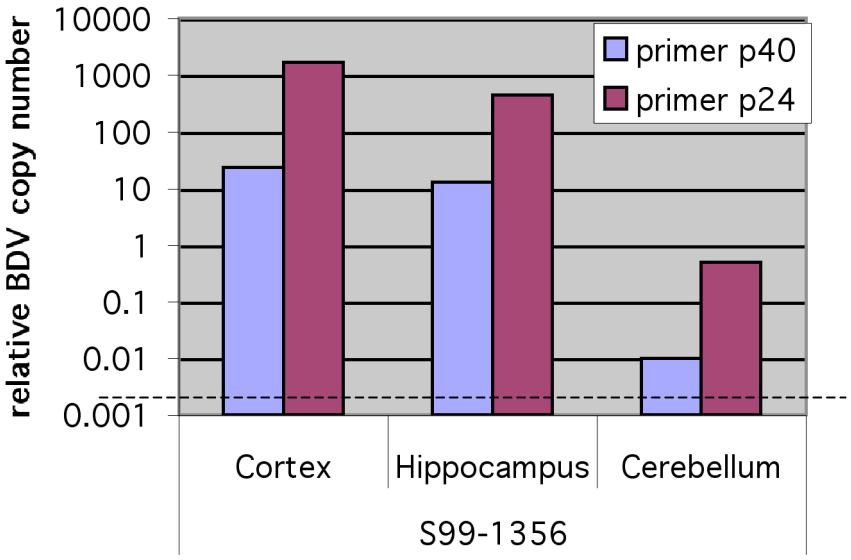


Fig. 12. Distribution of virus load in three different sheep brain sites of three BDV infected sheep (S03-0057, S02-1736 and S99-1356). The threshold for both primers is 0.005 (dashed line). x-axis: brain sites cortex, hippocampus and cerebellum. y-axis: relative BDV copy number.

Sheep	**Brain site**	**Real Time RT-PCR**		**Immuno-histochemical examination**
		Primer p40 **(Relative BDV copy number per cell)**	**Primer p24** **(Relative BDV copy number per cell)**	**p38/40**
S03-0057	Cortex (*)	74.4295436	254.158648	++
	Hippocampus	65.1576734	851.437847	+++
	Cerebellum	0.76140349	2.48873949	+
S02-1736	Cortex	0.07496186	0.54094983	+
	Hippocampus	0.32448315	5.46036848	+
	Cerebellum	0.008218	0.00594945	-
S99-1356	Cortex	23.6624731	1678.44101	+++
	Hippocampus	13.0346773	444.378386	+++
	Cerebellum	0.00985846	0.50818802	+ - ++
S02-0857	Threshold	0.00506513	0.0051369	-

Table 3. Results, normalised and calibrated, of the three BDV infected sheep. Threshold of the results of the negative control (sheep S02-0857). Values represent the average from three independent experiments, except those marked with an asterisk (*) (only one experiment). Immunohistochemical examination: No antigen (-) or low (+), moderate (++) and large amount (+++) of BDV antigen detected.

The threshold of 0.005 (both, p40 and p24) represented the average relative BDV copy number of three samples each from cortex, hippocampus and cerebellum from a BDV negative sheep, as diagnosed by immunohistochemical methods (see fig. 13).

Therefore, values below 0.005 were considered negative. Notably, virus loads in the three positive sheep were consistently lower in the cerebellum compared to cortex and hippocampus. Interestingly, a much lower BDV load was observed for sheep S02-1736 than for the other two; this result was also confirmed by the immunohistochemical data (see table 3).

Furthermore, C_T values generated with primers and probe for p40 were higher than those generated with primers and probe for p24, suggesting higher transcription levels for phosphoprotein p24 compared to the nucleoprotein p40.

4.2.2.2. Horses

Frozen brain samples of three horses suffering from BD were tested for the presence of BDV RNA as well (see figs. 13 and 14 and table 4). For one horse, S00-913, the same three brain tissues as from the sheep were analysed. However, for the other two horses, no cerebellum samples were available, but lobus piriformis instead. Consequently, lobus piriformis was used for the analysis in addition to tissues from the cortex and the hippocampus for these two horses. The negative threshold of 0.0004 (for both, p40 and p24) was calculated as for the sheep, using the C_T values from cortex, hippocampus and cerebellum of a BDV negative horse for the analysis. In the BDV positive horse S00-913, BDV load in the cortex and hippocampus were again higher than in the cerebellum, supporting the trend from the three sheep. In the other two cases, in contrast, lobus piriformis showed similar (S96-868) or even higher (S99-0598) BDV load than in the cortex or hippocampus. This may be explained by the fact that the lobus piriformis belongs to the bulbus olfactorius, which is assumed to be the host entry point for the virus. In general, the three horses showed higher maximum BDV load (approx. 10-fold) than the sheep, combined with a lower background (also approx. 10-fold). In accordance with the results of the three BDV infected sheep, transcription levels of p24 appear to be higher than those of p40.

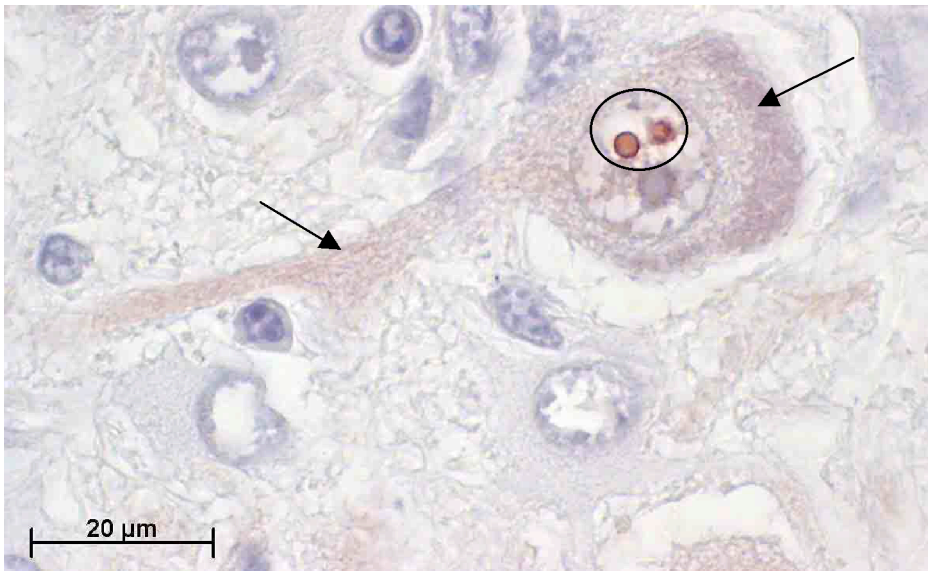
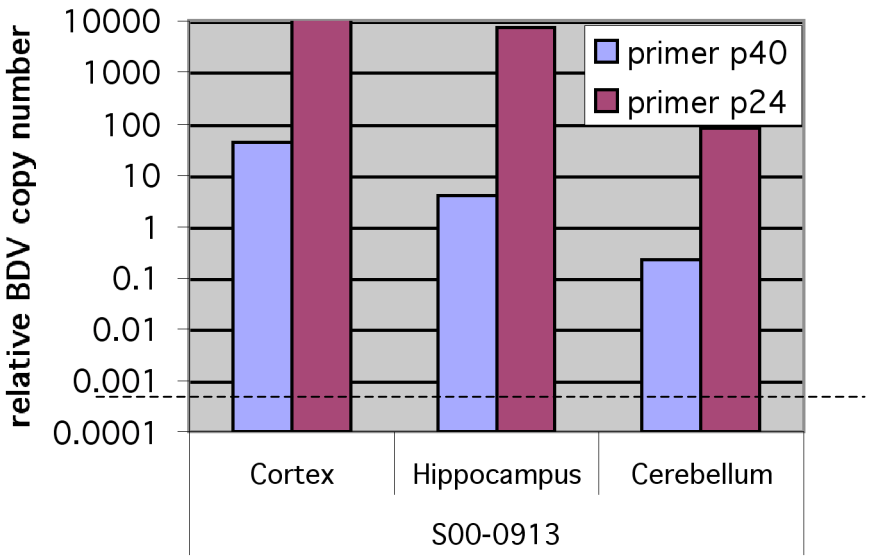


Fig. 13. Immunohistochemical examination. Hippocampus of a horse (S00-0913) suffering from BD, p38/40, magnification 100x. Circle: Intranuclear reaction (Joest-Degen inclusion body), arrow: Cytoplasmic reaction in a BDV infected neuron.



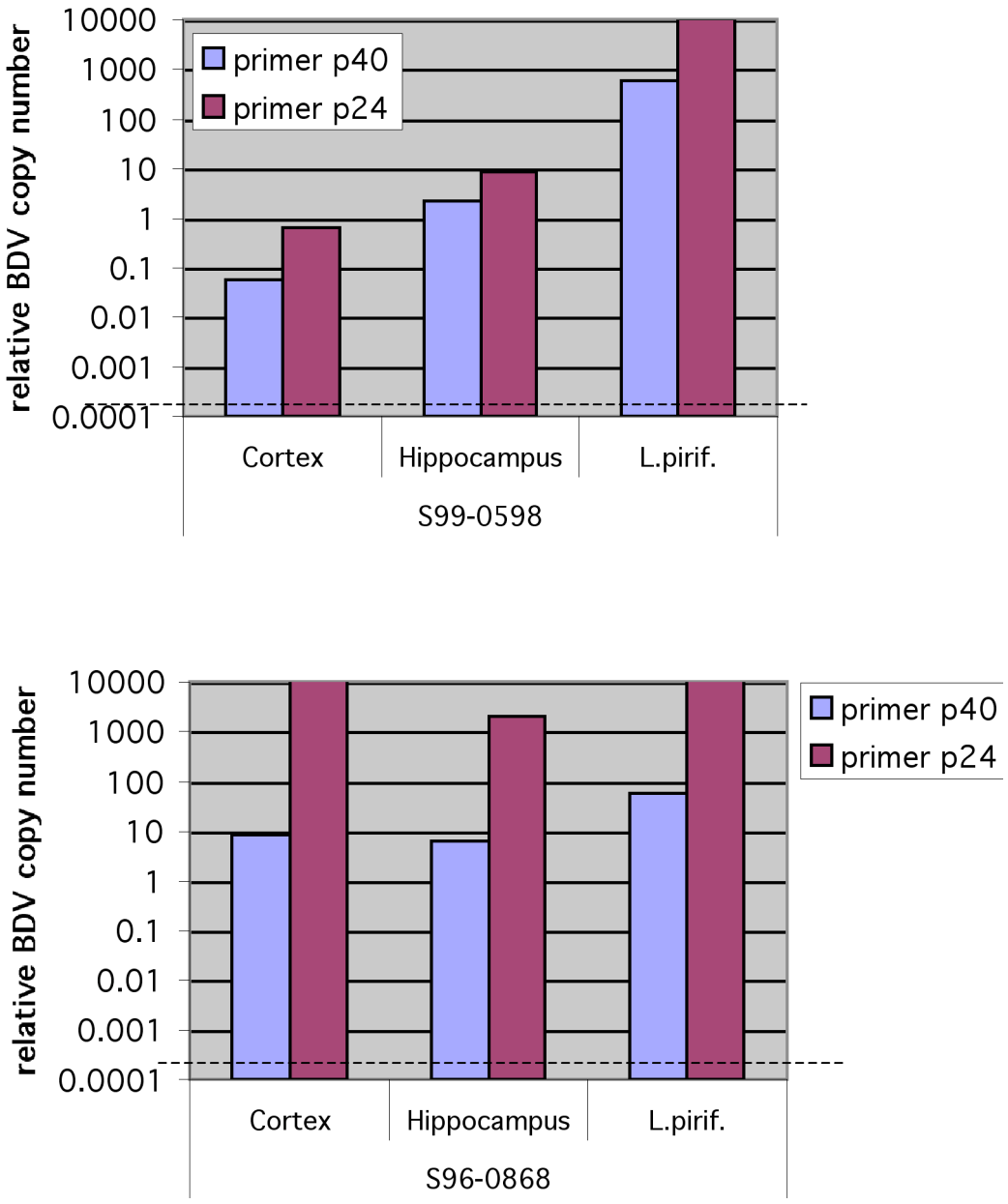


Fig. 14. Distribution of virus load in three different brain sites of three BDV infected horses (S00-0913, S99-0598 and S96-0868). The threshold for both primers is 0.0004 (dashed line). x-axis: brain sites cortex, hippocampus and cerebellum or lobus piriformis. y-axis: relative BDV copy number.

Horse	Brain site	Real Time RT-PCR		Immuno-histochemical examination
		Primer p40 **(relative BDV copy number per cell)**	**Primer p24** **(relative BDV copy number per cell)**	
S00-0913	Cortex (**)	43.6613061	10938.4734	+++
	Hippocampus (**)	3.89402325	7296.1528	+++
	Cerebellum	0.22276789	82.6800901	-
S99-0598	Cortex	0.05586269	0.6373934	-
	Hippocampus (**)	2.20686871	8.60967767	++
	L.piriformis	568.546009	10378.2222	Not available
S96-0868	Cortex	8.31192757	12798.0021	+++
	Hippocampus	6.29523072	2008.15664	+++
	L.piriformis	57.6033	14248.2219	Not available
S01-1038	Threshold	0.00038879	0.00039515	-

Table 4. Results, normalised and calibrated, of the three BDV infected horses. Threshold of the results of the negative control (horse S01-1038). Numbers represent the average from three independent experiments, except those marked with two asterisks (**) (only two experiments). Immunohistochemical examination: No antigen (-) or low (+), moderate (++) and large amount (+++) of BDV antigen detected.

4.2.3. Experimental infection of ticks

In six independent experiments, a total number of approx. 500 ticks were fed with a suspension containing 2 x 10^6 MDCK cell/ml (approx. 75% of these cells were infected), of which they ingested not more than 10 to 30µl (i.e., 2 – 6 x 10^4 cells) as described by Burgdorfer et al. (1957) [118]. A total number of 150 Ixodes ticks were included in this study (93 ticks, fed with BDV infected MDCK cells and 46 control ticks, fed with non infected MDCK cells). Starting one day post-infection, at least 4 ticks (and at least two control ticks, fed with non infected MDCK cells; see threshold in figs. 15 and 16) were used per time point for RNA extraction and subsequent analysis of the virus load by TaqMan® PCR. While we were not able to keep the ticks of experiments 1-4 alive for more than 10 to 15 days post-infection, individual animals of experiment 5 and 6 survived up to 30 days.

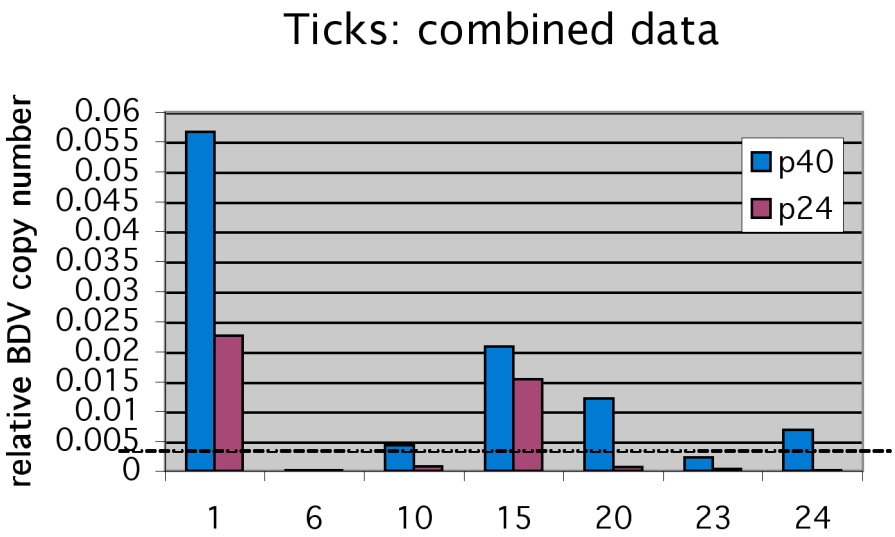


Fig. 15. Average virus load in BDV infected ticks at different time points post infection. The threshold for both primers is 0.004 (dashed line). x-axis: days post infection. y-axis: relative BDV copy number.

The combined data in figure 15 clearly show that virus load per tick gradually decreased over time after ingestion of BDV-positive MDCK cells, reaching background levels between 10 and 25 days post-infection. Furthermore, the relative BDV copy number of the nucleoprotein p40 is clearly higher than that of p24. In figure 16 the trend of all experiments is shown. The threshold of both primers is 0.004. Approx. 15 days post infection the signal is below the threshold and should therefore be considered negative. The difference in virus load between single experiments and individual ticks may be explained by the extremely low overall BDV levels, which were likely at the limits of detection.

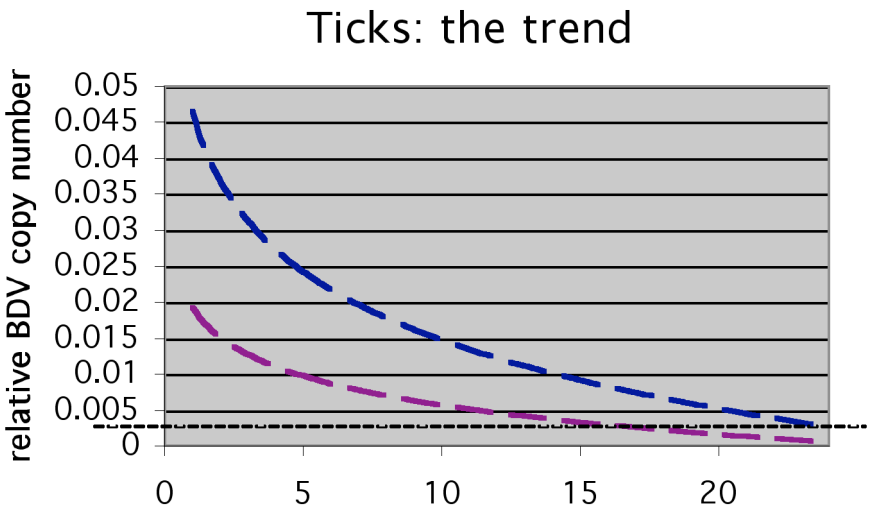


Fig. 16. Average virus load in BDV infected ticks at different time points post infection: trendline. The threshold for both primers (blue p40, red p24) is 0.004. Approx. 15 days post infection the signal is below the threshold, i.e., the ticks are considered to be negative. x-axis: days post infection. y-axis: relative BDV copy number.

5. Discussion

In the present thesis we have successfully established TaqMan® Real Time RT-PCR for the detection of Borna Disease virus from various sources.

Although a number of PCR-based diagnostic methods for the detection of BDV already exist, the establishment of Real Time RT-PCR has brought a number of advantages. For example, the use of fluorescent dye-labelled probes increases the sensitivity of detection. But the major advantage is the determination of the C_T value within the logarithmic phase of amplification reaction rather than an endpoint determination of conventional PCR. Hence, the majority of diagnostic PCR assays reported to date have been used in a qualitative, or "yes/no" format. A main problem facing the diagnostic application of PCR is the production of false-positive results attributable to contaminating nucleic acids, particularly in the form of previously amplified material (carry-over). Any contaminant, carried over from the previous PCR procedure or from a strongly positive sample (contamination) may be multiplied and give false-positive results. In the TaqMan® system, the problem of carry-over is significantly reduced due to the real-time measuring principle, so described as "closed" or homogenous system, which makes it unnecessary to open the tubes after amplification and requires no post-PCR manipulation [119, 120]. Furthermore, cDNA from a former amplification is being destroyed by the Uracil-N-Glycosylase provided in the TaqMan® Universal Master Mix (Applied Biosystems, Foster City, CA, USA). This enzyme recognises the dUTP of any possible former cDNA product and destroys them in the first stage of the PCR. Therefore, it is not surprising that Real Time RT-PCR has gained increasing interest in virology, including studies on flaviviruses, hepadnaviridae, herpesviruses, orthomyxoviruses, parvoviruses, papovaviruses, paramyxoviruses, picornaviruses, retroviruses and TT virus [120].

TaqMan® Real Time RT-PCR was also used to investigate viral dynamics in the central nervous system of neonatally BDV infected gerbils [121]. With this exception, all previous studies have used conventional PCR approaches for the diagnosis of BDV, i.e., the results were qualitative and with only a limited value for quantitation. However, the paralleled investigation of the same brain sites of the chosen animals by immunohistochemical examination showed, that the results are

comparable and that immunohistochemistry was almost as sensitive as our PCR results. Anyhow, the investigation with Real Time RT-PCR allows an exact and objective evaluation of the results whereas the analysis of immunohistochemical examination by asterisks remains subjective and certain diversity in interpretation cannot be excluded.

For the establishment of TaqMan® Real Time RT-PCR, we initially optimised the PCR reaction with series of dilutions of primers and probes for *p40* and *p24* and subsequently determined the specificity by using a number of other RNA viruses as a template. The primer and probe sequences for *p40* and *p24*, respectively, were designed based on the consensus sequences of the four BDV strains Borna V, Borna HE/80, Borna H1766 and Borna No/98 (see section 4.1.1. Consensus primers). However, it should be noted that strain No/98, which is only approx. 80% identical to the other three BDV strains, might be missed with this primer/probe combination, since No/98 differs in one nucleotide position compared to forward primer of p40, the probe and the reverse primer of p24, respectively (see fig. 4). To confirm that we can indeed detect No/98 or other BDV variants, samples specifically infected with Borna No/98 should be used as a template. The TaqMan® output file from this experiment (see section 4.1.3. Specificity, fig. 5.) demonstrated that the primers specifically amplified BDV, while amplification of other templates yielded only background C_T values (C_T = 40). This initial experiment also confirmed our setting of the threshold cycle (C_T) at $\Delta Rn = 0.02$, since all reactions with virus templates other than BDV showed a C_T value of 40 while amplification of BDV resulted in a C_T value of 17. We then measured the molecular sensitivity of the PCR reaction by using a dilution series of two plasmids containing the *p40* ORF as a template. These experiments demonstrated that the system was able to detect between 1 and 10 plasmid molecules per reaction and thus were extremely sensitive.

The previous study by Watanabe et al. (2001) applying TaqMan® PCR for the detection of BDV used the absolute standard curve method by synthesising p40 and p24 cDNA from *in vitro* transcribed RNAs and diluting it in uninfected host cell tissues [121]. In contrast, we chose the relative standard curve method to compare relative virus load in different cell types. Specifically, we generated relative standard curves by using persistently infected MDCK cells as a template for a dilution series and host cell 18s rRNA as an endogenous control (see section 4.1.4.2. Generation of

relative standard curves using infected MDCK cells, fig. 8 to 10). These standard curves were then validated by measuring the cellular sensitivity to detect BDV in infected MDCK cells. Here, it was consistently possible to detect one BDV infected MDCK cell in 10^6 non-infected cells. Since the cell numbers were normalised to 10^6, relative BDV copy numbers per cell (or: relative virus load) should actually be multiplied by the factor obtained from the ratio of BDV-infected cells to non infected cells in which the former were diluted to get the relative virus load per cell. For example, the relative virus load of approx. 10 in 10'000 BDV infected MDCK cells should be multiplied by 100 (10^6 divided by 10'000), which results in a relative virus load per cell of approx. 1'000. In a recent study, RT-PCR was performed to estimate the copy number of genomic BDV RNA per infected host cell [122]. Specifically, either RNA extracted from BDV infected MDCK cells, or as an internal standard *in vitro* transcribed RNA from plasmid DNA containing the p40 ORF (allowing the estimation of an absolute virus load), was used as a template for the reaction. Applying this method, the absolute virus load per cell was estimated approx. 100 per cell. Our calculated relative virus load of approx. 1000 per cell was therefore 10 times more sensitive.

Having established and validated the method, we applied TaqMan® Real Time RT-PCR to detect BDV in brain tissues of the two major hosts, i.e., horses and sheep. The brain sites cortex and hippocampal formation were chosen according to the predilection sites of BDV in the brain. As expected, the relative virus loads in cortex and hippocampus were clearly higher than in the cerebellum, which is usually also less affected by the infection. Interestingly, the relative virus loads in lobus piriformis were higher than in cortex and hippocampus. This could be explained by the fact, that lobus piriformis belongs to the olfactory bulb, which is a potential entry point of the virus. However, since lobus piriformis is in close proximity to the hippocampal formation, there is a certain possibility that samples could include parts of hippocampal formation as well. But the data obtained by TaqMan® Real Time RT-PCR generally supported the immunohistochemical examination of higher virus load in cortex and hippocampus compared to the cerebellum. For future studies, it could be very interesting to measure absolute virus loads in different brain locations and hosts during the course of infection by establishing the absolute standard curve method.

An interesting observation made from the TaqMan® data is the different p24:p40 ratios seen in the various cell types. Specifically, infected MDCK cells and tissue samples from BDV infected horses and sheep showed a high p24:p40 ratio, while experimentally infected ticks had higher p40 values than p24. It should be noted, however, that the used random primers amplify both, genomic and mRNA, and therefore p40 and p24 levels reflect the sum of these two RNA populations. It has been known since a while that p40 and p24 specific antibodies are the major BDV components since they can also be detected in the sera of infected animals [123]. In addition, they are the main products in BDV infected cultured cells and were detected in brain samples from BDV infected animals by immunohistochemical techniques and western blot [124,125]. Interestingly, the ratio between p24 and p40 appears to change in the course of infection in that acutely infected cells show a higher level of p40 antigen, while persistently infected cells show higher levels of p24 [29]. Moreover, other studies demonstrated that virus replication was optimal when p40 antigen was in a 30-fold excess over p24, while the ratio of the transcript levels alone did not have an effect on polymerase activity [30-32]. Taken together, these studies strongly suggested that the stochiometry of p40 and p24 is critical for the regulation of the BDV replication cycle. While all these studies used in vitro systems, our data demonstrate for the first time similar p24:p40 ratios in sheep and horses naturally infected with BDV, which might argue for a persistent infection. The data are also in accordance with previous evidence that a specific ratio between the p40 and p24 homologues of other Mononegavirales including Paramyxoviruses is necessary for viral replication in infected cells [126-128]. Assuming that the high p24:p40 ratio (N:P) in cells persistently infected with BDV (and other viruses) would be highly unfavourable for virus replication (specifically also for incoming viruses), control of polymerase activity with a well-balanced ratio of the polymerase complex components P and N could be a strategy for superinfection exclusion [31]. Hence, it could be very fruitful for future experiments to determine these ratios during the course of infection in cell lines, experimentally infected mammalian hosts as well as potential vectors (see also below) as a potential means to measure the stage of infection.

As a second application of the TaqMan® Real Time RT-PCR, we chose to experimentally infect a potential vector and monitor the virus load over time. The choice of the tick *Ixodes ricinus* was based on the fact that it occurs in the endemic

BD areas, is a known vector of other virus-based diseases and has previously been proposed as a vector for the transmission of BD-like encephalomyelitis [70]. We used capillary artificial feeding of ticks which was first described by Burgdorfer and co-workers [118] and since then used and advanced by different groups to study ticks as a vector for Borrelia, Ehrlichia, Rickettsia, Escherichia coli and Bacillus subtilis [129-133]. It is obvious from our six independent experiments that infected cells could be detected over a period of one through 24 days post feeding. However, in the course of this time the virus load per tick decreased close to background or threshold level of 0.004 (see section 4.2.3. Experimental infection of ticks, figs. 15 and 16), indicating that the infection was probably not transmitted from the infected cells to the ticks. The differences in the detected relative BDV copy number may be explained by the irregular feeding habits during artificial feeding, since it was nearly impossible to get uniformly infected ticks for experimental studies. In addition, BDV copy numbers and 18s rRNA used for calibration were measured per whole tick, i.e., differences in BDV load in various tissues/organs could not be detected with this approach. Still, it is theoretically possible that BDV accumulates in a specific subcompartment of the tick. This would also explain the reversed p40:p24 ratios compared to the data from sheep and horse tissues, and argue for BDV replication in ticks, i.e. an acute phase BDV infection. Altogether, these observations do not necessarily argue for or against ticks being vectors for BDV, but our experiments indicate that the newly established Real Time RT-PCR may provide a useful tool for detecting such vectors in endemic areas. Possible alternative strategies to test the general hypothesis that transmission of BDV is arthropod-based could include random testing of arthropod samples from the endemic areas for the presence of BDV. It is still controversial whether viraemia occurs in BDV affected animals, which would be essential for an incorporation of the virus during blood feeding on hosts.

In summary, we present here the successful establishment of TaqMan® Real-Time PCR for the detection of Borna Disease virus. The primers detect BDV with high specificity and the approach also proved highly sensitive to detect both plasmids containing the p40 ORF and BDV from infected MDCK cells. In addition, Real Time RT-PCR allowed the relative quantification of virus load from different sheep and horse brain sites. These data were in good agreement with previous immunohistochemical studies using the same samples. Furthermore, our data

demonstrate that the Real Time RT-PCR approach is a valuable diagnostic and epidemiologic tool, which allows for comparative studies using infected samples from various sources, e.g., different tissues, body fluids and excretions (i.e., liquor, serum, urine) and detecting possible vectors in endemic areas.

6. References

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7. Abbreviations

A	Adenine
AEC	Aminoethyl Carbazole
Approx.	Approximately
BD	Borna Disease
BDV	Borna Disease Virus
C	Cytosine
cDNA	Complementary DNA
CNS	Central nervous system
CSF	Cerebro spinal fluid
C_T	Threshold cycle
DNA	Deoxdyribonucleic acid
dUTP	2'-deoxyuridine 5'-triphosphate
ΔR_n	Increase in fluorecence of the reporter dye
FAM	Reporter dye, 6-carboxyfluorescein
G	Guanine
G-protein	Glycoprotein
HE	Haemalaun-eosin
H_2O	Water
H_2O_2	Hydrogen peroxide
L-protein	Polymerase
M-protein	Matrix protein
MDCK	Madin and Darby canine kidney cells
NaN_3	Sodium azide
NNS RNA	Negative nonsegmented single-stranded
N-protein	Nucleoprotein (N)
nt	Nucleotide
ORF	Open reading frame
PBS	Phosphate buffer solution
PEDV	Porcine epidemic diarrhoea virus
PCR	Polymerase chain reaction
PI2	Parainfluenzavirus 2
PI3	Parainfluenzavirus 3
P-protein	Phosphoprotein (P)
RNA	Ribonucleic acid
mRNA	Messenger RNA
RT	Room temperature
RT PCR	Reverse transcription polymerase chain reaction
T	Thymine
T_M	Temperature
TAMRA	Quencher dye, 6-carboxytetramethylrhodiamine
Taq	Thermus aquaticus

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Annex

MDCK+	p40				p24				18s rRNA		
Number of cells	1. Result	2. Result	average normalized	**average calibrated**	1. Result	2. Result	average normalized	**average calibrated**	1. Result	2. Result	average normalized
10000.00	23.14	23.22	296583.65	**8.04159**	22.45	23.02	7407.89	**20.08470**	16.99	16.22	36881.21
10000.00	19.51	19.48	378431.7	**10.28718**	19.38	20.24	6326113.40	**17.19440**	12.96	13.98	367917.18
10000.00	17.89	17.82	11759571	**2.36309**	18.73	18.65	13578143	**2.72853**	10.19	9.47	4976349.70
1000.00	25.92	25.63	49576.59	**0.94743**	25.20	25.24	119946.15	**2.29222**	16.33	15.85	52327.54
1000.00	23.63	23.91	198118.12	**42.61532**	24.54	24.13	230168.51	**49.50937**	19.06	19.88	4648.99
1000.00	24.42	24.22	135160.55	**0.53582**	26.47	25.54	71816.78	**0.28470**	12.95	20.08	252251.12
100.00	30.58	30.93	1590.508	**0.03086**	30.62	30.48	2530.39	**0.04909**	16.56	15.74	51547.20
100.00	28.42	28.36	8096.7059	**0.01252**	29.75	29.44	5078.22	**0.00785**	12.44	12.78	646670.25
100.00	33.21	34.37	360.7561	**0.00471**	34.37	34.16	171.99	**0.00225**	15.30	15.84	76578.35
10.00	34.11	33.41	203.70829	**0.00597**	34.68	33.35	229.82	**0.00673**	17.22	16.26	34150.00
10.00	32.89	32.57	405.7201	**24.44721**	33.37	33.80	284.02	**17.11403**	26.23	40.00	16.60
10.00	34.78	34.56	105.8145	**0.00205**	37.00	37.25	21.70	**0.00042**	15.13	25.02	51715.95
1.00	36.94	35.63	38.160139	**0.00181**	40.00	40.00	2.69	**0.00013**	16.37	40.00	21039.99
1.00	36.10	40.00	20.961712	**0.00002**	40.00	38.62	5.01	**0.00001**	40.00	11.15	924371.36
1.00	40.00	38.04	6.4634635	**0.00010**	40.00	40.00	2.69	**0.00004**	15.62	15.92	65382.85
0.10	40.00	40.00	2.651597	**0.00003**	40.00	40.00	2.69	**0.00003**	15.99	15.17	77909.67
0.10	35.44	40.00	32.310561	**0.00044**	40.00	40.00	2.69	**0.00004**	17.47	14.85	72781.93
0.10	40.00	40.00	2.651597	**0.00006**	40.00	40.00	2.69	**0.00006**	15.93	16.96	42661.72

Table A. TaqMan® results of BDV infected MDCK cells diluted in non infected MDCK cells.

Sheep	Brain site	P40				P24				18s r RNA		
		1. Result	2. Result	average normalized	**average calibrated**	1. Result	2. Result	averaga normalized	**average calibrated**	1. Result	2. Result	average normalized
S03-0057	Cortex (1)	26.54	26.01	36516.068	**2738.494**	25.11	25.32	120716.76	**9053.06**	40.00	40.00	13.33436
	Cortex (2)	14.47	14.72	116666266	**74.42954**	13.96	14.09	398386704	**254.1586**	10.89	10.75	1567472.6
(BDV)	Cortex (3)	19.50	19.33	4155070.7	**311606.3**	15.97	15.97	97299747	**7296919**	40.00	40.00	13.33436
	Hippoc. (1)	23.14	22.97	333737.82	**84.64315**	19.68	20.01	5922402.8	**1502.05**	19.08	19.08	3942.8805
	Hippoc. (2)	23.11	23.51	280239.04	**1.203572**	22.13	22.37	1034328	**4.442235**	13.42	13.48	232839.53
	Hippoc. (3)	22.65	22.41	481319.97	**109.6263**	20.02	20.37	4600514.9	**1047.822**	19.46	18.55	4390.552
	Cereb. (1)	29.36	29.50	4019.5837	**1.421788**	28.05	27.97	15909.41	**5.627399**	19.54	19.54	2827.1336
	Cereb. (2)	28.28	28.28	8913.581	**0.132659**	26.99	27.23	30631.209	**0.455876**	15.19	15.14	67191.929
	Cereb. (3)	28.67	28.64	6876.4493	**0.729764**	28.29	28.28	13031.244	**1.382943**	17.53	18.34	9422.8362
S02-1736	Cortex (1)	28.81	29.11	5583.0704	**0.112207**	28.09	28.09	15007.608	**0.30162**	16.25	15.13	49756.754
	Cortex (2)	28.61	28.75	6757.335	**0.045746**	27.72	27.26	23496.922	**0.159071**	14.00	14.16	147713.4
(BDV)	Cortex (3)	26.98	26.88	22740.211	**0.66932**	25.25	26.54	81732.991	**0.240568**	12.86	13.00	339750.75
	Hippoc. (1)	25.34	25.29	69545.877	**0.209107**	22.28	22.36	979912.59	**2.946356**	12.69	13.29	332584.62
	Hippoc. (2)	27.09	27.22	19429.053	**0.469993**	23.62	23.92	344770.57	**8.340073**	15.35	16.59	41339.035
	Hippoc. (3)	24.75	25.36	84599.341	**0.294349**	21.65	21.89	1464268.4	**5.094677**	13.21	13.11	287411.44
	Cereb. (1)	40.00	40.00	2.657339	**0.000353**	35.30	36.35	59.466859	**0.007897**	19.99	17.43	7530.3521
	Cereb. (2)	36.92	38.57	14.734242	**0.024196**	40.00	40.00	2.6949909	**0.004426**	22.37	21.21	608.96563
	Cereb. (3)	40.00	36.52	16.236401	**0.000106**	33.34	34.31	250.54124	**0.00163**	13.94	14.11	153752.6
S99-1356	Cortex (1)	22.98	24.04	256492.45	**13.11569**	18.39	17.98	19781147	**1011.505**	16.67	17.10	19556.159
	Cortex (2)	20.01	19.52	3314493.7	**27.65392**	15.51	15.66	128776428	**1074.424**	14.06	14.76	119856.21
(BDV)	Cortex (3)	23.41	23.80	228375.75	**30.21782**	17.99	18.02	22290496	**2949.394**	18.08	18.29	7557.6528
	Hippoc. (1)	31.76	31.92	757.49627	**0.892086**	27.15	26.60	36896.973	**43.44527**	21.01	21.44	849.12963
	Hippoc. (2)	25.30	24.20	111462.11	**16.73734**	19.86	19.87	57955858	**870.3158**	18.10	18.67	6659.4887
	Hippoc. (3)	34.08	32.46	329.05638	**21.47461**	29.08	29.47	6425.9746	**419.3667**	28.00	28.08	15.323044
	Cereb. (1)	32.82	31.14	813.83796	**0.003981**	27.56	27.14	25944.658	**0.126893**	13.68	13.58	204452.32
	Cereb. (2)	33.32	32.98	308.1799	**0.020505**	27.70	27.76	19482.1	**1.296232**	17.14	17.33	15029.791
	Cereb. (3)	32.35	31.83	648.92571	**0.00509**	28.19	28.41	12931.292	**0.101433**	14.07	14.53	127485.48
S02-0857	Cortex (1)	40.00	40.00	2.657339	**0.001183**	40.00	40.00	2.6949909	**0.0012**	19.56	20.24	2245.9756
	Cortex (2)	40.00	40.00	2.657339	**0.000349**	40.00	40.00	2.6949909	**0.000354**	18.17	18.17	7618.0989
(control)	Cortex (3)	40.00	40.00	2.657339	**0.000505**	40.00	40.00	2.6949909	**0.000512**	19.15	18.33	5266.3745
	Hippoc. (1)	40.00	40.00	2.657339	**0.010071**	40.00	40.00	2.6949909	**0.010214**	22.85	22.85	263.85563
	Hippoc. (2)	40.00	40.00	2.657339	**5.88E-06**	40.00	40.00	2.6949909	**5.96E-06**	13.95	11.85	452088.15
	Hippoc. (3)	40.00	40.00	2.657339	**1.29E-05**	40.00	40.00	2.6949909	**1.15E-05**	13.46	13.41	235367.19
	Cereb. (1)	40.00	40.00	2.657339	**0.022785**	40.00	40.00	2.6949909	**0.023108**	23.14	27.81	116.62691
	Cereb. (2)	40.00	40.00	2.657339	**0.005611**	40.00	40.00	2.6949909	**0.005691**	22.35	21.76	473.58032
	Cereb. (3)	40.00	40.00	2.657339	**0.220051**	40.00	40.00	2.6949909	**0.223169**	38.75	36.20	12.075994

Table B. TaqMan® results from three BDV infected and one non infected sheep.

Horse	Brain site	P40				P24				18s r RNA		
		1. Result	2. Result	average normalized	**average calibrated**	1. Result	2. Result	averaga normalized	**average calibrated**	1. Result	2. Result	average normalized
S03-0913	Cortex (1)	24.19	24.13	150636.59	**0.08953**	14.39	14.48	295874408	**175.8505**	11.28	11.28	1682533.8
	Cortex (2)	32.63	33.06	376.56213	**28.1829**	24.42	24.63	198964.69	**14891.04**	27.93	27.15	13.36137
(BDV)	Cortex (5)	24.71	24.86	97910.298	**59.13971**	19.28	18.62	11565701	**6985.907**	20.96	20.72	1655.5761
	Hippoc. (1)	30.58	31.02	1548.3221	**6.845851**	20.44	20.96	3222228.4	**14246.97**	23.33	23.89	226.16941
	Hippoc. (2)	24.57	24.16	132023.5	**0.942195**	16.90	16.97	48389808	**345.3375**	14.72	14.70	140123.26
	Hippoc. (5)	32.91	33.14	329.91615	**78.97237**	24.68	23.88	246914.55	**59104.19**	29.09	29.09	4.1776149
	Cereb. (1)	29.17	28.71	5605.2871	**0.003415**	21.69	22.19	1310979.5	**0.79876**	11.27	11.36	1641268.9
	Cereb. (2)	37.20	37.75	15.4588	**0.144844**	28.90	29.34	7208.7492	**67.54345**	24.22	25.18	106.72758
	Cereb. (5)	36.84	36.58	25.86675	**0.520045**	28.92	28.70	8938.0829	**179.6981**	25.77	25.58	49.739452
S99-0598	Cortex (1)	28.48	28.42	7767.8269	**0.013134**	27.69	26.99	26668.184	**0.045092**	13.18	12.38	591410.84
	Cortex (2)	29.39	29.45	3973.3505	**0.050632**	27.10	27.63	25839.022	**0.329266**	15.51	15.51	78474.501
(BDV)	Cortex (5)	29.98	30.45	2323.5362	**0.103821**	26.82	27.08	34416.671	**1.537821**	17.77	16.86	22380.15
	Hippoc. (1)	20.31	20.00	2412199.6	**0.760214**	19.57	19.56	7202266.6	**2.269823**	10.44	10.37	3173052.1
	Hippoc. (2)	22.20	22.17	589726.91	**3.653523**	21.07	21.08	2413052.1	**14.94953**	14.54	14.49	161413.21
	Hippoc. (5)	29.19	28.96	5058.0662	**348.3916**	25.74	26.43	66116.461	**4553.997**	27.27	27.48	14.518336
	L. pirif. (1)	19.16	19.18	4737851	**3.116492**	19.05	18.75	11726527	**7.713544**	11.43	11.41	1520251.4
	L. pirif. (2)	20.80	20.80	1535788.1	**11.31617**	19.05	19.27	9687625.3	**71.38149**	14.39	15.25	135716.21
	L. pirif. (5)	18.68	18.80	6382745.8	**1691.205**	15.64	15.79	117206239	**31055.57**	19.62	19.78	3774.0809
S96-0868	Cortex (1)	19.11	19.26	4695177.1	**2.319828**	14.59	14.65	258699687	**127.8202**	11.83	10.52	2023933.7
	Cortex (2)	30.62	30.66	1709.7224	**19.5058**	20.98	20.36	3317350.4	**37846.82**	26.12	24.25	87.652016
(BDV)	Cortex (5)	24.60	23.54	171141.63	**3.110159**	17.60	18.44	23076097	**419.3622**	15.81	16.22	55026.656
	Hippoc. (1)	23.65	24.96	150442.96	**0.281931**	16.92	16.99	47694006	**89.37894**	12.57	13.24	533615.7
	Hippoc. (2)	23.39	23.13	281656.63	**8.502898**	15.56	15.83	119308864	**3601.801**	16.59	16.82	33124.782
	Hippoc. (5)	22.18	21.87	662427.28	**10.10086**	15.32	15.37	153020102	**2333.29**	15.68	15.84	65581.256
	L. pirif. (1)	20.56	21.01	1570591.4	**4.181665**	15.82	15.61	117372074	**312.5006**	13.23	13.48	375589.95
	L. pirif. (2)	25.36	24.65	86527.639	**6.478982**	17.13	17.08	42777928	**3203.109**	18.25	17.71	13355.129
	L. pirif. (5)	21.58	22.24	731756.11	**162.1493**	15.08	15.21	177035055	**39229.06**	19.13	19.87	4512.8553
S01-1038	Cortex (1)	40.00	40.00	2.651597	**2.73E-06**	40.00	40.00	2.6949909	**2.78E-06**	12.10	11.98	970940.1
	Cortex (2)	40.00	40.00	2.651597	**0.000387**	40.00	40.00	2.6949909	**0.000393**	19.17	18.63	6856.6548
(control)	Cortex (5)	40.00	40.00	2.651597	**0.001772**	40.00	40.00	2.6949909	**0.001801**	21.68	20.51	1496.1159
	Hippoc. (1)	40.00	40.00	2.651597	**3.16E-05**	40.00	40.00	2.6949909	**3.21E-05**	15.65	15.22	83865.206
	Hippoc. (2)	40.00	40.00	2.651597	**0.000114**	40.00	40.00	2.6949909	**0.000116**	18.06	16.66	23234.875
	Hippoc. (5)	40.00	40.00	2.651597	**115.2094**	40.00	40.00	2.6949909	**117.0948**	36.19	36.35	0.0230155
	Cereb. (1)	40.00	40.00	2.651597	**0.000121**	40.00	40.00	2.6949909	**0.000123**	17.38	17.17	21903.645
	Cereb. (2)	40.00	40.00	2.651597	**0.000453**	40.00	40.00	2.6949909	**0.000461**	19.36	18.87	5847.8705
	Cereb. (5)	40.00	40.00	2.651597	**0.000228**	40.00	40.00	2.6949909	**0.000232**	24.00	17.20	11613.641

Table C. TaqMan® results fromthree BDV infected and one non infected horse.

Ticks (part I.)		p40				p24				18s rRNA	
Experiment	Day	1. Result	2. Result	average normaluzed	average calibrated	1. Result	2. Result	average normaluzed	average calibrated	Result	calibrated
I.	1	35.40	37.05	42.037108	0.613639	36.25	38.19	25.364541	0.3702603	25.23	68.504627
	1	33.77	33.72	199.98263	0.0046167	34.22	33.29	262.30852	0.0060555	16.33	43317.566
	1	35.08	34.50	99.069202	0.0175234	37.50	36.92	20.774651	0.0036746	19.14	5653.547
	1	35.40	34.83	79.085441	0.0073132	35.50	36.07	58.261354	0.0053876	18.24	10814.065
	6	36.13	34.87	65.177323	0.00002401	35.40	36.30	57.334537	0.00002112	10.62	2714402.7
	6	35.47	36.44	45.872866	0.00001176	38.93	36.47	20.291675	5.203E-06	10.12	3899695.4
	6	32.34	33.08	422.30533	0.0001734	32.99	33.00	430.19708	0.0001767	10.77	2434822.7
	6	40.00	40.00	2.651597	0.0002068	40.00	40.00	2.6949909	0.0002102	18.01	12821.726
	10	37.27	37.80	14.811986	0.00001139	39.95	39.34	3.5703851	2.745E-06	11.63	1300890.5
	10	34.85	33.72	148.30258	0.00005027	35.86	35.32	66.953545	0.00002269	10.50	2950300.5
	10	35.11	33.85	131.90053	0.0188402	37.73	36.47	24.340746	0.0034767	18.84	7001.0195
	10	37.18	34.72	60.271661	0.0042319	37.98	40.00	7.1662139	0.0005032	17.86	14242.291
II.	1	32.25	32.62	498.49435	0.000435	32.25	33.39	530.51385	0.0004629	11.81	1145950.9
	1	30.73	30.00	2133.5276	0.0033123	30.16	29.95	3627.1333	0.0056311	12.61	644123.75
	1	32.02	33.02	494.35272	0.0003683	32.07	32.10	831.55266	0.0003428	10.78	2426016.7
	1	34.70	34.16	126.71974	0.0005155	34.25	34.60	153.95746	0.0001725	12.16	892462.73
III.	1	36.51	35.80	38.9506759	0.00034424	37.17	37.25	20.3333224	0.0001797	15.01	113151.036
	1	29.59	29.48	3671.64175	0.01126474	30.16	30.27	3223.53817	0.00988994	13.55	325941.2
	1	34.53	34.89	103.427817	0.00030825	35.48	35.90	61.8125287	0.00018423	13.51	335527.231
	1	33.46	33.42	246.895854	0.00036833	34.88	34.49	127.781246	0.00019063	12.55	670314.356
	1	31.21	31.80	959.889198	0.00051546	32.80	31.62	829.930195	0.00044567	11.14	1862188.34
IV.	1	29.83	29.49	3388.58842	0.01129982	31.34	31.73	1250.71331	0.00417071	13.66	299879.85
	1	32.46	32.66	454.609063	0.01230866	34.92	34.53	124.132905	0.00336093	16.55	36934.0747
	1	30.85	29.95	2116.37297	0.00163277	31.99	31.95	903.811275	0.00069729	11.64	1296185.6
	1	33.42	32.54	355.073055	0.00270485	36.39	35.22	61.3429717	0.00046729	14.80	131272.775
V.	6	34.34	34.77	115.500842	459.983767	39.59	40.00	3.160815	12.5879913	32.97	0.25109765
	6	35.13	35.93	60.4717648	3.44740929	40.00	38.71	4.77705791	0.27233328	27.11	17.54122
	8	40.00	40.00	2.651597	0.00011009	40.00	40.00	2.6949909	0.00011189	17.14	24085.0003
	8	35.75	36.59	39.0045578	8.6682E-05	37.20	38.65	13.8181213	3.0709E-05	13.10	449973.012
	8	40.00	36.05	21.6521044	6.1562E-05	40.00	38.90	4.33619854	1.2329E-05	13.44	351709.506
	10	40.00	40.00	2.651597	0.00181844	40.00	40.00	2.6949909	0.0018482	21.01	1458.16955
	10	32.74	31.92	553.148976	0.00010017	34.71	35.70	92.4557174	1.6743E-05	9.64	5521954.71
	10	40.00	40.00	2.651597	6.6533E-07	40.00	39.27	3.63370447	9.1175E-07	10.09	3985401.4
	13	36.18	40.0	19.9055259	0.14283518	40.0	40.0	2.6949909	0.01933832	24.25	139.360108
	13	40.0	38.63	4.74306628	0.01416179	39.16	37.94	8.46551086	0.02527622	23.04	334.91993
	13	35.38	37.08	42.271478	0.01171786	40.0	40.0	2.6949909	0.00074706	19.76	3607.4395
	15	29.38	28.62	5494.56256	0.00075007	31.82	31.75	1033.60621	0.0001411	9.25	7325391.53
	15	40.0	40.0	2.651597	0.0428398	40.00	40.0	2.6949909	0.04354088	25.37	61.8956474
	15	40.0	36.03	21.9350111	0.01910667	40.00	40.0	2.6949909	0.00234749	21.34	1148.02883
	19	36.15	36.47	34.1743294	0.04307753	40.00	40.00	2.6949909	0.0033971	21.85	793.321551

Ticks (part II)		p40				p24				18s rRNA	
Experiment	Day	1. Result	2. Result	average normaluzed	average calibrated	1. Result	2. Result	average normaluzed	average calibrated	Result	calibrated
	19	35.02	40.00	42.7457839	2.6343E-05	40.00	40.00	2.6949909	1.6608E-06	11.33	1622662.38
	19	31.89	32.70	566.155122	0.00012651	35.39	36.34	57.0476635	1.2747E-05	9.93	4475340.89
	20	33.30	33.54	251.169605	0.00012011	37.39	35.75	38.1736225	1.8255E-05	10.98	2091113.74
	20	40.00	36.17	20.0343778	1.7867E-05	40.00	40.00	2.6949909	2.4034E-06	11.84	1121307.24
	20	40.00	40.00	2.651597	1.9165E-06	40.00	40.00	2.6949909	1.9479E-06	11.55	1383538.81
	23	36.19	34.96	61.625413	0.00597348	37.60	38.73	11.042197	0.00107034	18.31	10316.5064
	23	31.40	31.60	945.788406	0.00150605	33.93	35.11	155.788389	0.00024807	12.64	627992.369
	23	37.04	36.12	29.6208287	0.00033858	40.00	39.72	2.99789349	3.4267E-05	15.36	87485.3803
	23	36.62	36.90	25.0042447	0.00178773	40.00	38.51	5.31155081	0.00037976	17.89	13986.5962
	27	40.00	40.00	2.651597	0.0001855	40.00	40.00	2.6949909	0.00018854	17.86	14293.9883
	27	40.00	40.00	2.651597	0.00014395	40.00	40.00	2.6949909	0.0001463	17.51	18420.5635
	27	40.00	40.00	2.651597	0.00058291	40.00	40.00	2.6949909	0.00059245	19.44	4548.90807
	35	40.00	40.00	2.651597	840.485713	40.00	40.00	2.6949909	854.240425	39.01	0.00315484
	35	40.00	40.00	2.651597	0.06961535	40.00	40.00	2.6949909	0.07075462	26.04	38.0892585
	35	40.00	40.00	2.651597	0.00013389	40.00	40.00	2.6949909	0.00013608	17.41	19804.9702
VI.	1	38.36	38.33	27.0002626	0.40572951	40.00	40.00	2.6949909	0.04049729	25.27	66.547446
	1	35.30	36.16	133.995295	0.03032291	40.00	40.00	2.6949909	0.00060987	19.48	4418.94551
	1	34.18	36.98	131.243428	0.00817655	38.71	38.43	7.63005395	0.00047536	17.70	16051.1988
	10	36.10	36.54	96.7876057	0.0032806	37.93	39.81	7.57953027	0.00025691	16.86	29503.0046
	10	36.58	40.00	21.4547938	0.00173506	37.68	37.89	13.4412639	0.001087	18.06	12365.4765
	10	40.00	40.00	8.6883368	0.00813683	40.00	40.00	2.6949909	0.00252392	21.44	1067.77933
	10	35.01	35.23	229.487864	0.00980848	38.87	40.00	4.40183926	0.00018814	17.18	23396.8905
	20	32.82	34.02	616.505591	0.0231276	36.68	37.57	22.7473071	0.00085334	17.00	26656.7041
	20	38.22	35.98	117.382028	0.00610121	39.38	38.76	5.41876	0.00028165	17.45	19239.1411
	20	38.01	37.15	56.2326933	0.00201976	40.00	40.00	2.6949909	9.6798E-05	16.94	27841.2815
	20	40.00	40.00	8.6883368	0.01619692	40.00	40.00	2.6949909	0.00502404	22.39	536.419216
	20	35.90	34.67	297.202478	0.02334818	40.00	40.00	2.6949909	0.00021172	18.02	12729.149
	20	36.44	34.81	265.27328	0.03886397	39.94	40.00	2.75483083	0.0004036	18.88	6825.68656
	24	35.40	37.34	76.6693461	0.00332474	38.01	40.00	7.04116419	0.00030534	17.20	23060.2434
	24	34.42	34.39	394.872599	0.00722911	40.00	40.00	2.6949909	4.9338E-05	16.01	54622.5723
	24	32.55	32.19	1707.83569	0.00043794	36.62	36.67	30.6050184	7.8481E-06	10.12	3899695.38
	24	35.30	34.37	370.842394	7.0142E-05	39.63	39.76	3.36482341	6.3643E-07	9.70	5287009.26
	24	35.06	34.23	410.579237	0.00088638	38.31	36.48	21.8237355	4.7114E-05	13.06	463206.858
	24	37.33	34.76	266.766135	0.02989101	39.53	40.00	3.24134137	0.00036319	18.51	8924.62726
	29	33.90	34.01	519.768453	0.00513963	38.90	37.99	8.7654361	8.6675E-05	15.16	101129.544
	29	36.16	35.70	155.313195	1.28828647	40.00	40.00	2.6949909	0.02235432	24.45	120.557965
	29	33.90	33.85	569.110352	0.00170232	37.18	38.81	13.5755754	4.0607E-05	13.51	334313.727
	29	40.00	40.00	8.6883368	0.93718243	40.00	40.00	2.6949909	0.29069984	27.99	9.27069962
	29	36.36	37.17	66.7060418	0.04743433	40.00	40.00	2.6949909	0.00191639	21.06	1406.28189
	29	40.00	40.00	8.6883368	0.0008361	40.00	40.00	2.6949909	0.00025934	18.30	10391.5368

Ticks (part III)		p40				p24				18s rRNA	
Experiment	Day	1. Result	2. Result	average normaluzed	average calibrated	1. Result	2. Result	average normaluzed	average calibrated	Result	calibrated
	30	28.59	28.00	28975.824	11.2915033	30.55	40.00	1264.91738	0.49292192	20.23	2566.16176
	30	28.06	28.01	30362.9525	0.04152407	30.93	31.62	1541.80272	0.00210855	12.43	731213.262
	30	33.30	32.93	1031.1282	0.00331612	37.37	36.14	31.1060371	0.00010004	13.61	310944.534
	30	33.19	33.44	779.861432	0.13111927	35.43	40.00	38.2295424	0.00642759	19.07	5947.72534
	30	40.00	40.00	8.6883368	1.0736E-05	40.00	39.22	3.71800213	4.5942E-06	12.29	809289.406
	30	31.83	31.78	2329.98828	0.00435147	35.54	36.56	50.3294063	9.3995E-05	12.86	535448.301
	35	40.00	38.36	23.7463583	42.9257008	40.00	40.00	2.6949909	4.87166796	31.88	0.55319675
	35	40.00	40.00	8.6883368	324.755587	40.00	40.00	2.6949909	100.73428	36.06	0.02675346
	35	40.00	40.00	8.6883368	4.85536909	40.00	40.00	2.6949909	1.50606219	30.26	1.7894287
	35	40.00	40.00	8.6883368	31.950348	40.00	40.00	2.6949909	9.91051558	32.86	0.27193246
	35	40.00	40.00	8.6883368	3183.47929	40.00	40.00	2.6949909	987.467213	39.21	0.0027292
	35	40.00	40.00	8.6883368	4.54881312	40.00	40.00	2.6949909	1.41097315	30.17	1.9100228
	35	40.00	40.00	8.6883368	24.7928301	40.00	40.00	2.6949909	7.69036159	32.51	0.35043748

Table C. (Part I-III) TaqMan® results of BDV infected ticks (*Ixodes ricinus*). Six independent experiments.